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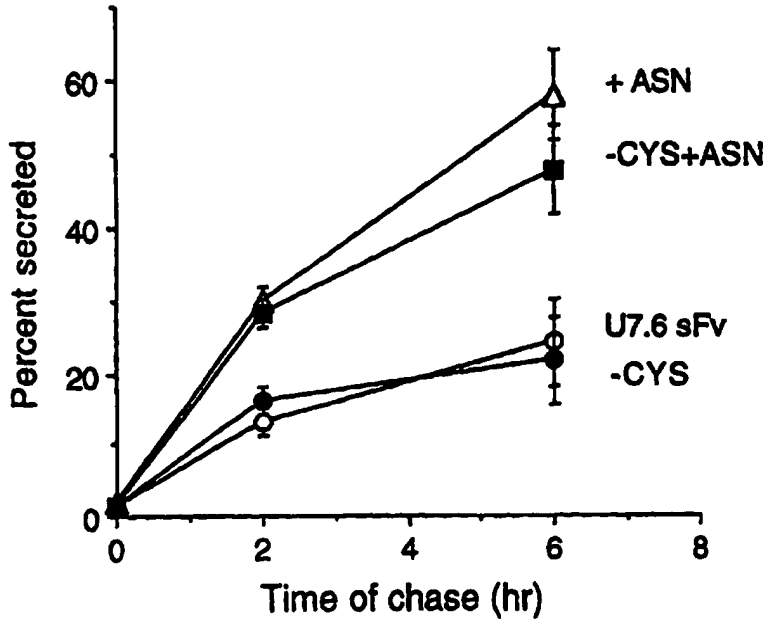

(57) Abstract

The invention relates to a method of producing single-chain Fv molecules in eukaryotic cells, and to secretable sFv proteins having at least one non-naturally occurring glycosylation site. The single-chain Fv molecules produced by this method are biologically active and capable of being secreted from eukaryotic cells into the cell culture medium.

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METHOD OF PRODUCING SINGLE-CHAIN Fv MOLECULES

Background of the Invention

Single-chain Fv (sFv) proteins are genetically engineered molecules that consist of the two variable domains of an antibody or T cell receptor connected by a polypeptide linker and that contain the antigen binding function of the parental protein in a single 30 kD polypeptide chain. (Huston, J.S., et al., Proc. Natl. Acad. Sci. U.S.A. 85:5879-5883 (1988); Bird, R.E., et al. Science 242:423-426 (1988); Huston, J.S., et al., Meth. Enzymol. 203:46-88 (1991)).

The Fv portion of an antibody is the smallest fragment to bear the complete antigen-binding site. It is a 25 kD heterodimer consisting of the N-terminal variable (V) domains of the heavy (H) and light (L) chain. (Inbar, D., et al., Proc. Natl. Acad. Sci. U.S.A. 69:2659-2662 (1972); Hochman, J., Biochemistry 15:2706-2710 (1976); Hochman, J., et al., Biochemistry 12:1130-1135 (1973)). More recently, a genetically engineered single-chain Fv (sFv) with antigen binding activity has been produced by connecting the C-terminus of one V domain to the N-terminus of the other with a peptide linker. Huston, J.S., et al., Proc. Natl. Acad. Sci U.S.A. 85:5879-5883 (1988); and Bird, R.E., et al., Science 242:423-426 (1988) Since then, sFv proteins have been produced from a large number of different antibodies (Huston, J.S., et al., Intern. Rev. Immunol. 10:195-217 (1993); Winter, G. and Milstein, C. Nature 349:293-299 (1991)) and initial studies (Kurucz, I., et al., Proc. Natl. Acad. Sci. U.S.A. 90:3830-3834 (1993); Novotny, J., et al., Proc. Natl. Acad. Sci. USA 88:8646-8650 (1991); Soo Hoo, W.F., et al., Proc. Natl. Acad. Sci. USA 89:4759-4763 (1992); Ward, E.S., J. Mol. Biol.

224:885-890 (1992)) have described the production of sFv analogues of T cell receptors (TcR), cell surface molecules that are highly homologous to immunoglobulins (Hedrick, S.M., et al., Nature 308:153-158 (1984); Davis, M.M. and Bjorkman, P.J., Nature 334, 395-402 (1988)).

Most sFv proteins have been generated in bacteria, often as insoluble, cytoplasmic inclusion bodies. Protein from inclusion bodies is not active and must be solubilized, renatured in vitro and oxidized to form parent disulfide bonds, (Huston, J.S., et al. Methods Enzymol. 203:46-78 (1991)). Alternatively the introduction of an N-terminal leader sequence can direct sFv into the periplasmic space of bacteria by a secretion process wherein the leader sequence is removed (Holland, I.B., et al., Methods Enzymol. 182:132-143 (1990)) and protein folding is accomplished, aided by enzymes that catalyze disulfide bond formation (Bardwell, J.C.A., et al., Cell 67:581-589 (1991)) and cis-trans isomerization of proline residues (Hayano, T., et al., Biochemistry 30:3041-3048 (1991)). However, even with these enzymes, secreted sFv proteins sometimes exist as insoluble aggregates in the periplasmic space, which must be solubilized and refolded in vitro (Johnson, S. and Bird, R.E., Methods Enzymol. 203:88-98 (1991); George, A.J.T., et al., J. Immunol. 152, in press (1994)). Knappik, A., et al. (Bio/Technology 11:77-83 (1993)) have recently attempted to overcome this problem by overexpressing protein disulfide isomerase and prolyl cis-trans isomerase in the piroplasm of bacteria. However, neither enzyme induced a significant change in folding efficiency of sFv proteins when expressed either alone or together with the other enzyme.

A different approach to produce active sFv would be to use the more sophisticated refolding machinery that is located in the endoplasmic reticulum (ER) of mammalian

cells. The potential benefit of this approach could be substantial, since the ER not only contains enzymes that catalyze specific isomerization steps but it also contains a number of proteins (e.g., chaperones) that aid in the folding process and prevent the secretion of incorrectly folded proteins (Gething, M.J. and Sambrook, J. Nature 355:33-45 (1992); Pelham, H.R., Annu. Rev. Cell Biol. 5:1-23 (1989); Hurtley, S.M. and Helenius, A., Annu. Rev. Cell Biol. 5:277-307 (1989)). A number of sFv fusion proteins have been expressed in or on the surface of mammalian cells. Examples include an anti-HIV-KDEL fusion protein, or anti-HIV sFv alone, that remains bound in the ER (Marasco, W.A., et al. Proc. Natl. Acad. Sci. USA 90:7889-7893 (1993)) and anti-tumor sFv proteins fused to TcR-ζ or $Fc_{\epsilon}RI$-γ that trigger cell mediated cytolysis (Eshhar, Z., et al. Proc. Natl. Acad. Sci. USA 90:720-724 (1993); Hwu, P., et al. J. Exp. Med. 178:361-366 (1993); Stancovski, I., et al. J. Immunol. 151:6577-6582 (1993)). However, production of this class of proteins by mammalian cells is generally very low, varying from a few micrograms to a few milligrams, if production is possible at all. (Davis, S.J., et al., J. Biol.Chem. 265:10410-10418 (1990); Traaunecker, A. et al., EMBO J., 10:3655-3659 (1991)).

To date, it is not certain what the rate-limiting step, or steps, in the efficient expression and secretion of sFv proteins in mammalian cells may be, nor has it been apparent how to induce, or increase existing production levels.

Summary of the Invention

The present invention relates to a method of producing single-chain Fv molecules in mammalian cells and single-chain Fv molecules produced by this method. The single-chain Fv molecules produced by this method are

capable of being secreted from mammalian cells into the cell culture medium, thus greatly facilitating their isolation and purification. Importantly, the single-chain Fv molecules produced by this method are secreted as correctly folded, biologically active binding molecules capable of reacting with their respective ligands, thus eliminating the need of further in vitro manipulation to remove bacterial endotoxin and to further purify or to refold these molecules to recover biological activity.

The method described herein is based on the finding that, in the secretion of single-chain Fv molecules from mammalian cells, their exit from the endoplasmic reticulum can be rate-limiting and that glycosylation of the single-chain Fv molecules can enhance the rate of secretion.

More specifically, the parent nucleic acid sequence encoding a single-chain Fv molecule is modified by oligonucleotide-directed mutagenesis of the coding sequence to include one, or more, non-naturally occurring glycosylation site, or sites. As used herein, the term single-chain Fv molecule includes novel analogs of the single-chain Fv. These sFv analogs include, for example, the sFv' and the (sFv')2 molecules wherein a cystine-containing peptide is fused to the sFv carboxy terminus. Another example includes a BiBABS molecule (V_H-V_L-V_H-V_L) wherein two sFv molecules are linked together. Descriptions of additional single-chain Fv molecules encompassed by this invention are found in Huston, J.S., et al. Cell Biophysics., 24 in press (1994), the teachings of which are incorporated herein by reference. Also encompassed by this invention are chimeric multivalent protein analogs described in WO 93/23537, the teachings of which are also incorporated herein by reference, and antibody fragments such as Fv, Fab and

Fab' fragments (Better, M. and Howitz A.H., Enzymol. 178:476-796 (1989)). As used herein, the term non-naturally occurring glycosylation site means a glycosylation site not encoded by the parent single-chain Fv nucleic acid sequence. The novel glycosylation site, or sites, are incorporated into the parent single-chain Fv nucleic acid sequence at an appropriate amino acid residue, or residues contained within the sequence. Preferably, the novel glycosylation site, or sites, are either N-linked (asparagine-linked) or O-linked (serine- or threonine-linked) glycosylation sites. The parent sFv coding sequence is modified in such a manner (e.g., by insertion, deletion, or substitution of nucleotides) so as to result in the consensus amino acid sequence Asn-X-Ser/Thr, which leads to N-linked glycosylation, or Ser/Thr, which leads to O-linked glycosylation. In a preferred embodiment, the novel glycosylation site(s) is(are) located in a region of the sFv protein product that is not buried within the folded protein (e.g., exposed on the protein surface at a β-turn, in a loop, or within a linker sequence).

The modified sFv nucleic acid construct (also referred to herein as the sFv construct) is introduced into a vector capable of expressing the glycosylated sFv protein construct in a eukaryotic cell. In a preferred embodiment, the eukaryotic cell is a mammalian cell. The term vector, as used herein means any nucleic acid sequence comprising a nucleic acid sequence of interest, competent to be incorporated into a eukaryotic host cell resulting in the expression of the nucleic acid sequence of interest. Vectors can include, for example, linear nucleic acid sequences, plasmids, cosmids, phagemids, and extrachromosomal DNA. Specifically, the vector can be a recombinant DNA vector. Also as used herein, the term expression, or gene expression, is meant to refer to the

production of the protein product of the nucleic acid sequence of interest, including transcription of the DNA and translation of the RNA transcript. The eukaryotic host cell can be any mammalian cell capable of expressing protein, including for example, immortalized mammalian cells such as COS-7 cells, 293 cells, myeloma, Chinese hamster ovary (CHO) cells. The host cells can also be cultured yeast cells.

The vector is transfected into the eukaryotic cell, for example, by calcium phosphate precipitation, and the transfected cell is maintained under conditions sufficient for propagation of the cells, expression of the sFv construct within the cell and secretion of the sFv protein product into the cell culture medium. For example, if a mammalian cell is the transfected host cell, the cell is cultured in suitable culture medium and under an atmosphere conducive for growth of the cell. As the host cell grows, the vector integrates into the host cell genome and expresses the sFv construct within the host cell resulting in the sFv protein product. Importantly, this sFv protein product contains at least one novel, engineered glycosylation site that was not present in the parent sFv molecule. This new glycosylation site signals the attachment of oligosaccharide (carbohydrate) chains to the sFv protein, which takes place within the endoplasmic reticulum (ER) of the eukaryotic cell. Under the conditions described herein, glycosylated sFv proteins are secreted from the ER at an increased rate relative to the rate of secretion of parent sFv proteins. Importantly, the secretable, glycosylated sFv molecules described herein, typically exhibit biological activity and properties which have not been previously accessible through bacterial (prokaryotic) expression or secretion. Additionally, glycosylation of CDRs in some antibodies can be important

to improve binding, especially to carbohydrate antigens (Wright, A. and Morrison, W., in SPRING SEMINARS IN IMMUNOLOGY 15:259-273 (1993)),. Thus specific or random introduction of glycosylation sites into CDRs of sFv proteins, as described by the present invention can be of value.

The present invention further relates to modified secretable sFv proteins having one, or more, non-naturally occurring glycosylation site(s), and to the DNA sequences encoding these proteins. More specifically, these proteins have at least one N-linked or O-linked glycosylation site that is not encoded by the parent sFv protein. These modifications are also referred to herein as post-translational modifications. Although these sFv proteins are modified to contain non-naturally occurring glycosylation sites, they retain the same specificity of binding as exhibited by the parent, unglycosylated sFv protein.

Thus, as a result of the method described herein, post-translationally modified sFv molecules capable of specifically binding ligand can be successfully produced in and secreted from eukaryotic cells.

Brief Description of the Drawings

Figure 1 is a diagrammatic representation of the sFv gene constructs.

Figure 2A shows the nucleic acid sequence (SEQ ID NO: 14) an encoded amino sequence (SEQ ID NO: 15) of the V_H region of U7.6 sFv.

Figure 2B shows the nucleic acid sequence (SEQ ID NO: 16) and the encoded amino acid sequence (SEQ ID NO: 17) of the V_L region of U7.6 sFv.

Figure 3A is an electrophoretic gel showing the distribution of sFv in COS-7 cells and culture supernatant at different times in a pulse chase experiment.

Figure 3B is a graphic representation showing the rate of secretion of the different sFv molecules.

Figure 4 is an electrophoretic gel showing the processing of N-linked carbohydrate during sFv secretion.

Figure 5A is an electrophoretic gel showing the presence of U7.6 sFv mutants in cells and supernatant in a pulse chase experiment.

Figure 5B is a graphic representation showing the densitometric analysis of the data shown in Figure 5A. sFv present in the supernatant is plotted as percent of total immunoprecipitation material.

Figure 6 is an electrophoretic gel showing the preferential secretion of glycosylated forms of sFv proteins from tunicamycin-treated cells.

Figure 7 is an electrophoretic gel showing that secreted sFv proteins specifically bind their antigen.

Figure 8 is a graphic representation showing the results of experiments demonstrating the inhibition of binding of U7.6 sFv mutant proteins by DNP-hapten.

Figure 9 is a graphic representation showing the results of experiments demonstrating that OKT9-sFvs produced in mammalian cells and bacteria dissociate from K562 cells with similar rates.

Detailed Description of the Invention

The invention described herein relates to a method of producing single-chain Fv molecules in eukaryotic cells and single-chain Fv molecules produced by this method. Specifically, the invention relates to a method of producing single-chain Fv proteins that are capabl of being secreted from mammalian cells directly into the

cell culture medium. Importantly, these single-chain Fv proteins are secreted as correctly folded, biologically active binding molecules, capable of binding ligand with specificity.

The method described herein is based on the finding that, in the secretion of single-chain Fv proteins from mammalian cells, their exit from the endoplasmic reticulum can be rate-limiting and that glycosylation of the single-chain Fv protein can enhance rates of secretion.

A wide variety of proteins are secreted by vertebrate cells. In fact, some cells are highly specialized to secrete specific proteins, such as B-lymphocytes that secrete immunoglobulins. Ribosomes that synthesize secreted proteins are bound to the endoplasmic reticulum (ER). After synthesis these proteins are translocated into the lumen of the ER and then move in small transport vesicles through the Golgi complex and eventually exit the cell. This entire process is often termed protein maturation.

Specific maturation steps known to occur in the ER include proteolytic cleavage of leader sequences, addition and modification of carbohydrate residues, formation of disulfide bonds, and the folding of the nascent polypeptide chain into its correct three-dimensional structure. The first two processes occur very rapidly with all proteins, and for example, with antibodies, the formation of disulfide bonds occurs as the peptide passes into the lumen of the ER. (Bergman, L.W. and Kuehl, W.M., J. Biol. Chem. 254:8869-8876 (1979)).

In the case of antibodies, nascent heavy (H) and light (L) chains are known to bind to chaperones, proteins resident in the ER that facilitate the folding and assembly of H and L chains into functional

antibodies. Both chains associate with heavy chain binding protein (BiP or GRP78) (Knittler, M.R. and Haas, I.G. EMBO J. 11:1573-1581 (1992)) and GRP94 (Melnick, J., et al. J. Biol. Chem. 267:21303-21306 (1992)) during folding and assembly, and IP90, another putative chaperone, interacts with partial complexes of membrane immunoglobulin in the ER of B-cells (Hochstenbach, F., et al. Proc. Natl. Acad. Sci. U.S.A. 89:4734-4738 (1992)). Once antibodies have assumed their proper configuration they dissociate from the chaperones and proceed through the Golgi on their way to being secreted. In the case of sFv proteins, little is known about protein folding. One recent report discussed a non-secretable sFv (i.e., an sFv which was produced but not secreted by mammalian cells) that interacted with BiP. (Marasco, W.A., Haseltine, et al. Proc. Natl. Acad. Sci. USA 90:7889-7893 (1993)). Another report discussed that the V_H domain contributes to the binding of immunoglobulin heavy chains to BiP. (Pollak, R.A., et al. Proc. Natl. Acad. Sci. U.S.A. 84:9199-9203 (1987)).

Moreover, many antibodies, other secreted proteins and most cell surface proteins are glycosylated. That is, they have oligsaccharides covalently linked to amino acid residues. (Machamer, C.E. and Rose, J.K., J. Biol. Chem. 263:5948-5954 (1988)). Many potential functions have been suggested for these oligsaccharides, including assistance with polypeptide folding, prevention of intracellular aggregation, protection from proteolytic breakdown and signals for intracellular targeting and cellular recognition. (Olden, K. et al., Biochem. Biophys. Acta 650:209-232 (1982)). As described herein, it is now demonstrated that mammalian cells transfected with different sFv genes secrete the corresponding active sFv molecules at different rates and that glycosylation can affect these secretion rates.

A nucleic acid sequence encoding a single-chain Fv (sFv) protein can be modified to include one, or more non-naturally occurring N-linked, or O-linked glycosylation site(s). The unmodified nucleic acid sequence encoding the sFv protein is referred to herein as the parent sFv nucleic acid sequence. As used herein, the term non-naturally occurring glycosylation site means a glycosylation site that is not encoded for in the parent nucleic acid sequence. Thus, the modified sFv protein contains at least one glycosylation site that is not encoded by the parent nucleic acid sequence.

sFv proteins suitable for modification by the method described herein, include single-chain antibody proteins, such as U7.6, other Ig superfamily analogues, such as the T-cell receptor protein and chimeric derivatives of these sFv proteins. A detailed description of sFv molecules is found in U.S. Patents Nos. 5,091,513, issued February 25, 1992, and 5,132,405, issued July 21, 1992, the teachings of which are incorporated herein by reference. A description of chimeric single-chain protein analogues is found in International Patent Application, WO 93/23537, the teachings of which are incorporated by reference. References to nucleic acid sequences and constructions of sFv molecules are described, for example, in Huston, J.S., et al., Intern. Rev. Immunol. 10:195-217 (1993). The sFv proteins encompassed by this invention also include sFv fusion proteins where effector domains are fused to either chain terminus of the sFv, as described, for example, in Huston, J.S., et al., Meth. Enzymol. 203:46-88 (1991).

Nucleic acid sequences encoding single-chain Fv proteins, Ig superfamily analogues, chimeric proteins, sFv fusion proteins and other sFv protein species can be modified, as described in Example 1, by oligonucleotide-directed mutagenesis of the coding sequence to include

one, or more, glycosylation site(s) that are not present in the parent sequence. The modified nucleic acid sequence encoding an sFv protein containing at least one novel gylcosylation site that was not encoded by the parent nucleic acid sequence is referred to herein as the modified sFv nucleic acid sequence, or sFv construct.

N-linked (asparagine-linked) sites are the preferred sites of glycosylation contemplated by this invention. The parent sFv coding sequence is altered in such a manner (e.g., by addition, deletion, or substitution of nucleotides) so as to result in a nucleic acid sequence that encodes the consensus amino acid sequence Asn-X-Ser/Thr. This consensus sequence signals an N-linked glycosylation site.

However, O-linked glycosylation sites are also encompassed by this invention. If an O-linked glycosylation site is inserted, the parent sFv nucleic acid sequence is altered so as to result in a modified nucleic acid sequence that encodes the amino acid sequence Ser/Thr. This consensus sequence signals an O-linked glycosylation site.

The selection of the novel glycosylation site, or sites, is based on the combination of the primary nucleic acid sequence encoding the sFv molecule and the local tertiary structure in the parent protein, which can, for example, represent a β-turn or loop structure. (Aubert et al, Arch. Biochem. Biophys. 175:410 (1976)). The novel glycosylation site(s) is(are) located in a region of the protein that is not expected to be buried within the folded protein, nor sequestered at the V_H-V_L interface. That is, the novel glycosylation site is attached to an amino acid residue that is exposed on the protein surface. For example, the presence of an N-linked glycosylation site in the first framework region of V_H of two sFvs (OKT9 Ab-sFv, and U7.6Ab-sFv) increases their

rate of secretion without significantly altering their antigen binding affinities. If more than one glycosylation site is added, the sites can be constructed to be adjacent to each other, (e.g., attached to adjacent amino acid residues located within the amino acid sequence) or they can be interspersed at various/random positions within the sequence (e.g., attached to non-adjacent amino acid residues of the sequence).

The modified sFv nucleic acid construct is then introduced into a vector capable of expressing the modified construct in a host cell as described in Example 2. Such vectors, especially recombinant DNA vectors, are well known to those skilled in the art. These vectors supply a promoter and other elements necessary to express the construct in eukaryotic cells. Specifically, plasmid vectors are contemplated for use in the present invention, which include both prokaryotic sequences and mammalian transcription units, such as described in MOLECULAR CLONING: A LABORATORY MANUAL, 2d Ed. Sambrook, J., et al., eds., Cold Spring Harbor Laboratory Press, NY, (1989).

The vector is then introduced into a host cell by methods known to those of skill in the art. Introduction of the vector into the host cell can be accomplished by any method that introduces the construct into the cell, including, for example, calcium phosphate precipitation, microinjection, or electroporation. See, e.g., Cockett et al., Bio/technology, 8:662-667 (1990); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Ausubel, F.M., ed., John Wiley & Sons, NY (1989)). For example, as described in Example 2, a mammalian cell is used as the host cell. The mammalian cell can be transfected using the calcium phosphate method. The transfected host cell is maintained under conditions sufficient for propagation of the cell and expression of the sFv construct within the

cell. Host cells can include, for example, immortalized mammalian cells such as COS-7 cells, 293 cells, myeloma or Chinese hamster ovary (CHO) cell lines, and cultured yeast cells. Conditions sufficient for propagation of the cell and expression of the sFv construct include any type of culture system and media known to those of skill in the art suitable for the propagation of the host cell. Such culture systems include microcarrier or hollow fiber culture systems, as well as suspension systems, cultured under optimal conditions of media, atmosphere and temperature. As the host cell grows, the vector, for example, integrates into the host cell genome and expresses the sFv construct within the host cell resulting in the sFv protein product.

Importantly, the expressed sFv construct protein product contains at least one novel glycosylation site that is not encoded by the parent sFv sequence. Within the endoplasmic reticulum (ER) of the mammalian cell oligosaccharide (carbohydrate) chains are attached to the sFv protein at these glycosylation sites and the sFv protein is folded into its biologically active conformation. These correctly folded, glycosylated sFv proteins are then transported from the ER to the cell surface and secreted from the cell. Key to this method is the fact that, under the conditions described herein, sFv molecules with one, or more, non-naturally occurring glycosylation sites are secreted from the cell at a faster rate relative to the secretion rate of unglycosylated sFv molecules, and that these glycosylated sFv molecules retain their biological activity.

The secretion of sFv molecules into cell culture medium as biologically active, soluble proteins greatly facilitates their isolation and purification. Most current strategies used to produce sFv molecules have reli d on bacterial expression of fusion proteins.

(Huston, J.S., et al., Meth. Enzymol. 203:46-88 (1991)). sFv proteins produced in bacterial culture often contain bacterial endotoxin which contaminates the protein and requires further purification procedures to produce clinically acceptable proteins. Moreover, if periplasmic secretion methods do not produce active sFv, fusion proteins may be secreted or expressed in bacterial cells as insoluble inclusion bodies which require further solubilization and refolding to obtain biologically active proteins. In most cases, denaturants such as SDS, urea, or guanidine HCl have to be used to extract the inclusion body sFv protein. Subsequently, the protein has to be renatured (i.e., correctly folded) to achieve biological activity and this may prove difficult, if not impossible. (PRINCIPLES OF GENE EXPRESSION, 4th Ed., Old, R.W. and Primrose, S.B., eds., Blackwell Scientific Publications, Cambridge, MA (1989)). In the case of binding proteins, e.g., a protein such as an sFv protein, folding to achieve the correct three-dimensional conformation is a prerequisite of its biological activity.

In addition to correct folding to achieve biological activity, many different post-translational modifications have been described as necessary for active proteins. (PRINCIPLES OF GENE EXPRESSION, 4th Ed., Old, R.W. and Primrose, S.B., eds., Blackwell Scientific Publications, Cambridge, MA (1989)). Besides N- and O-linked glycosylation, these modifications include phosphorylation, acetylation, amidation, sulphuration, attachment of fatty acids and the formation of unusual amino acids. These post-translational modifications are not known to occur in bacteria. Some glycosylation has been reported in yeast and in baculovirus cells, however, the composition and sequences of the resulting oligosaccharide chains can differ significantly from

those found in mammalian cells. Thus, expression and secretion of sFv molecules in mammalian cells, as described herein, provides a rapid and efficient method of producing biologically active sFv molecules. The sFv protein produced by the described method can be isolated, purified and tested for biological activity using known laboratory techniques, such as described in Examples 3 and 4. For example, if the sFv protein is an antibody sFv, the biological activity can be tested by immunoprecipation, ELISA, or radioimmunoassay. Furthermore, testing biological activity of the sFv protein produced by the method described herein is greatly facilitated because the sFv protein is secreted in soluble form, directly into the cell culture medium. It is simply a matter of obtaining an aliquot of culture medium to test the activity of the sFv protein directly without further manipulation.

The present invention also relates to modified, secretable sFv proteins having one, or more, non-naturally occurring glycosylation site(s) and to the DNA sequences encoding these modified sFv proteins. Additional advantages are achieved by producing glycosylated sFv molecules. Non-glycosylated proteins can be subject to aberrant aggregation such as that mediated by non-covalent association (McCoutney et al., Protein Eng., (1994) or intermolecular disulfide bonding. (Machamer, C.E. and Rose, J.K., J.Biol.Chem. 263:5955-5960 (1988)). For example, as protein accumulates in the ER, cysteine residues in neighboring proteins can cross-link to form multimers. This results in large aggregates of protein which can be difficult to disassociate in order to further isolate and purify the protein. Alternate types of aggregation can involve non-covalent self-association, as for the first example, mediated by the bottom surface of an sFv, sFv analog or an antibody

fragment, which is diametrically opposite to the antigen combining site. This can result in an equilibrium mixture of monomers, dimers and even higher aggregates. Oligosaccharide chains positioned at specific amino acid residues in the proteins can play an important role in the prevention of aggregation and non-covalent association.

In clinical applications, the presence of oligosaccharide chains can protect the protein from proteolytic degradation, resulting in increased circulating half-life of the protein. (Goto, M, et al., Bio/Technology, 6:67-71 (1988)). The presence of an oligosaccharide chain, terminating in sialic acid, can also modify the in vivo biodistribution, pharmokenetics, elimination and/or renal uptake of a single-chain Fv molecule. For example, if the terminal sialic acid is removed, the remaining carbohydrate chain residue can be a residue recognized by hepatic receptors, thus signaling elimination. If a sialic acid residue is present, the signal for elimination can be blocked, again resulting in longer circulating half-life of the protein. Thus, the present invention provides a method of modifying the pharmokenetics of sFv proteins in the body.

The attachment of oligosaccharide chains can also decrease the antigenicity of the sFv molecule. For example, the linker sequence used in an sFv molecule to connect V_H and V_L could elicit an antigenic response to the sFv molecule. The addition of a glycosylation site within the linker can prevent the unwanted response, or decrease the severity of the response by masking the antigenic amino acid residues responsible for the response.

sFv Constructs

To evaluate sFv production in mammalian cells, COS-7 cells were transfected with vectors encoding four different sFv molecules; U7.6-sFv directed against the hapten DNP, OKT9-sFv against the human TfR, 2C11-sFv against the murine CD3-ϵ chain, and 2B4, a TcR-sFv that recognizes a cytochrome C peptide bound to I-E^k. These sFv molecules are described in detail in Example 1. Light chain leaders were used to direct newly-synthesized Ab-sFv proteins to the endoplasmic reticulum, and a c-myc peptide sequence was introduced at the 3' end of each Ab-sFv construct to facilitate detection of the proteins. The three Ab-sFv proteins contained a $(G_4\text{-}S)_3$ linker connecting the 3' end of V_L with the 5' end of V_H, as shown in Figure 1. OKT9-sFv is exceptional in that it has an N-linked glycosylation site located at position 19 in FR1 of V_H. The U7.6 antibody also differs from the other Ab-sFv proteins in that it has an extra cysteine in V_L, located in CDR3, 3 amino acid residues C-terminal to the second domain-forming cys, as shown in Figure 2A and 2B. The TcR-sFv construct contained a V_α leader sequence, V_α, and a linker consisting of 14 amino acid residues of C_α followed by two additional residues resulting from a Pst I site. This joined V_β and 7 C_β residues. The 2B4 sFv has three glycosylation sites, one in V_α, one in V_β and one in C_β, and two additional cysteine residues in the third framework portion of V_β.

Production and secretion of sFv molecules

Transfected COS-7 cells were labeled as described in detail in the Example 2, with [^{35}S]-met for 60 min and then chased with unfolded methionine for either 0, 2, or 6 h. The sFv secretion products were then precipitated from both the cell lysates and the negative supernatants using mouse antibody to the C-terminal myc peptide tag

and rabbit anti-mouse Ig bound to Protein A-Sepharose. Figure 3A and 3B shows the results of the production and secretion of sFv in COS-7 cells. Figure 3A shows the distribution of sFv in cells and culture supernatant at different times during a pulse chase experiment. COS-7 cells were transfected with plasmid DNA encoding the indicated sFv and 48 h later pulsed for 1 h with [^{35}S]-methionine- Cells were subsequently chased for 0 ("P" in the figure), 2 or 6 h as indicated. The sFv proteins were immunoprecipitated, subjected to SDS-PAGE under reducing conditions, and visualized by autoradiography. Figure 3B shows the rate of secretion of the different sFv molecules. Autoradiograms were quantified by densitometry. Total amounts of sFv in cell lysate and supernatant were determined and the percentage of total sFv present in the supernatant at each time point was calculated and plotted. Both OKT9 and 2C11 sFv proteins were secreted from the cells at similar rates, culminating in most sFv being present in the medium after a 6 h chase.

By contrast, the U7.6-sFv was secreted much more slowly, with only a minor amount being present in the medium after the 6 h chase. The 2B4 TcR-sFv was handled differently by the COS cells than the Ab-sFv proteins, as most of this sFv could be found in the supernatant, even after a 6 h chase. Data from three comparable independent experiments were quantified by densitometry and the percentages of total sFv in the supernatant were calculated and plotted (Figure 3B). The percentages of sFv present in the supernatant at various times are comparable for the 2C11 and OKT9-sFv proteins, resulting in about 70% secretion after a 6 h chase. At this time only 25% of the U7.6 sFv and 5% of the 2B4-sFv were secreted in corresponding experiments.

Exit from the ER is the rate limiting step in OKT9-sFv secretion

The transfer of OKT9 and 2B4-sFv proteins from the ER to the Golgi complex was followed by measuring the acquisition of endo H resistance that is conferred on N-linked oligosaccharides by additional changes in glycosylation that occur in the Golgi. Transfected COS-7 cells were pulse labeled and chased. sFv and immunoprecipitates were either treated or not treated with endo H prior to SDS-PAGE analysis, as described in Example 3. Figure 4 shows that the major fraction of cell-associated OKT9 sFv remained sensitive to endo H at all time points, while OKT9 sFv present in the medium was always endo H resistant. Endo H resistance was used to determine the localization of sFv in COS-7 cells at different times following pulse-labeling. Immunoprecipitates were either not treated (-) or treated (+) with endo H prior to analysis by SDS-PAGE. Samples indicated by "Cells" represent immunoprecipitates from cell-associated material at the designated times; samples indicated "Supe" are immunoprecipitates from culture supernatants 6 h after the pulse labeling.

The fact that most cell-associated OKT9-sFv was endo H sensitive indicated that it had not yet passed through the Golgi complex, and was therefore located in the ER. As a control, OKT9-KDEL sFv, which contains an ER retention signal, mostly remained cell associated and endo H-sensitive at all time points (Figure 4). The small amount of sFv reaching the medium from these cells was endo H-sensitive, suggesting that it was released directly into the medium from the ER, most likely as a result of cell death. The 2B4 TcR-sFv behaved quite differently from the OKT9. This protein remained cell-associated and endo H-sensitive at all time points (Figure 4), and was never secreted. Unlike the OKT9-KDEL

sFv, the glycosylation pattern of the 2B4-sFv changed with time.

Introduction of a glycosylation-site in the U7.6 sFv enhances its secretion rate

The U7.6 sFv was secreted much more slowly than the others antibody sFv constructs. In order to find the source of this defect, two mutations were introduced into the U7.6-sFv, as described in Example 1. First, the extra cysteine present in CDR 3 of V_L (-Cys in Figure 5A and 5B) was removed, and secondly an N-linked glycosylation site was introduced in the same position as the glycosylation site in OKT9 (+Asn). Figure 5A shows SDS-PAGE analysis of immunoprecipitates from cell lysates and culture supernatants from COS cells transfected with mutated U7.6 sFv proteins at different times during a pulse chase experiment. Figure 5B shows a densitometric analysis of the data shown in Figure 5A plotted as percent of immunoprecipitated material present in the culture supernatant.

A third construct incorporated both mutations (-Cys+Asn). COS-7 cells were transfected with the U7.6 mutants, and pulse chase experiments were performed. The data from three independent experiments were quantified and the percentages of total sFv in the supernatant were calculated and plotted in Figure 5B. The removal of the extra cysteine residue did not result in a change in secretion level. However, the introduction of a glycosylation site both in the presence (+Asn) and absence (-Cys+Asn) of the additional cysteine markedly enhanced the rate of sFv secretion; after 6 hr, 20-25% of the non-glycosylated sFv proteins were present in the medium, as compared with 50-60% of the glycosylated proteins. Endo H experiments indicated that U7.6 +Asn and U7.6 -Cys+Asn were indeed glycosylated and that the

rate limiting step in their secretion was the exit of the proteins from the ER, similar to OKT9-sFv.

To confirm the implication that glycosylation of the sFv proteins enhanced their rates of secretion, COS-7 cells transfected with the OKT9, U7.6+Asn and U7.6-Cys+Asn sFv constructs were treated with suboptimal concentrations of tunicamycin, as described in Example 2. Matched plates of transfected COS-7 cells were pretreated for 2 h, pulsed for 1 h, and chased for 2 h, all in the presence of 3 pg/ml tunicamycin. The sFv proteins were immunoprecipitated from cell lysates and analyzed by SDS-PAGE immediately after the pulse step (Cells/Pulse), and secreted sFv proteins were analyzed after a 2 h chase (Supe/Chase). On the right of Figure 6, the percentages of immunoprecipitated sFv proteins that were glycosylated are noted, as determined by densitometry, and based on the quotient of density of the higher Mr band divided by the sum of both bands' densities.

As shown in Figure 6, after pulsing with ^{35}S-methionine, the immunoprecipitated sFv proteins migrated as two bands corresponding to glycosylated and non-glycosylated forms. After a 2 hr chase, there was an enrichment of the glycosylated, relative to the non-glycosylated band in supernatants from all three transfectants. Thus, glycosylation accelerates the rate of secretion of both U7.6 and OKT9-sFv proteins.

Secreted antibody sFv proteins specifically bind antigen

To determine if secreted sFv proteins were active, the ability of radiolabeled material from the medium of transfected COS-7 cells to specifically bind antigen was tested as described in Example 4. Each radiolabeled sFv present in 6 h chase media was tested for antigen binding activity. For OKT9 and 2C11-sFv proteins, cells bearing the relevant antigen were incubated for 4 h at 4° C with

sFv in the presence or absence of an inhibiting antibody. The cells were subsequently washed, lysed, and bound sFv immunoprecipitated and analyzed by SDS-PAGE under reducing conditions. The reactivity of U7.6 sFv (wild type) was assessed by incubating DNP-Sepharose beads with the radiolabeled chase media for 16 h at 4° C in the absence or presence of 1 mM DNP-ε-aminocaproate. The beads were subsequently washed, boiled in SDS-loading buffer, and eluted material was analyzed by SDS-PAGE under reducing conditions and the bands were quantified by densitometry.

Figure 7 shows that all three sFv proteins were indeed active; OKT9-sFv bound to K562 cells, which express high levels of human TfR, binding was inhibited by intact OKT9 antibody, but not by W6/32, an antibody that recognizes MHC Class I molecules on these cells. Similarly, 2C11 sFv bound to 2B4 cells, which are $CD3^+$, and the binding was totally inhibited by 2C11 whole antibody but only slightly inhibited by H57, an antibody that recognizes the β chain of the TcR. Finally, U7.6 and the three U7.6 sFv mutants bound to DNP-Sepharose beads in the absence, but not in the presence of inhibiting hapten.

Sequential depletion experiments were done to determine the percentages of secreted sFv that had antigen binding activity. After two sequential incubations with DNP beads, 87-96% of the secreted U7.6-sFv constructs were specifically absorbed to the beads. Similar experiments using multiple sequential incubations of secreted OKT9-sFv with K562 cells indicated that the vast majority of this sFv was also functional, that is, specifically bound antigen.

To determine if the introduced mutations have any effect on the binding affinities of the U7.6-sFvs, the binding of ^{35}S-methionine labeled U7.6-sFv constructs to

DNP-beads was inhibited with increasing amounts of DNP hapten as described in Example 4. A shown in Figure 8, the greatest changes in affinity resulted from removal of cys 91_L: inhibition of binding required about 10 fold less hapten for the -Cys mutants than for the +Cys, suggesting that the affinity for hapten increased about 10 fold on removal of cys 91_L. Introduction of a glycosylation site at position 19 in FR' of V_H in the +Cys mutants had no significant effect on binding activity, while it caused an approximately 4-fold decrease in affinity in the -Cys mutants. Thus, introduction of an N-linked glycosylation site had only a small effect on the binding activity of U7.6-sFv.

The effect of glycosylation on the binding affinity of OKT9-sFv was assessed by comparing dissociation rates of bacterially produced (and therefore non-glycosylated) and refolded sFv with that of OKT9-sFv secreted from COS-7 cells. Figure 9 shows that the two products dissociate from K562 cells with very similar rates, suggesting that they have similar affinities for the TfR, as described in Example 5.

The amount of OKT9-sFv secreted by the transfected COS-7 cells was estimated by inhibiting its binding to the TfR with unlabeled OKT9 Fab, which binds with essentially the same affinity as the bacterially produced OKT9-sFv. By this assay, it is estimated that a COS cell supernatant contained about 0.17 μg/ml of ^{35}S-methionine labeled OKT9-sFv.

Thus, as described herein, transfected mammalian cells can secrete active antibody sFv at different rates, and the rate of secretion can be governed by glycosylation. In pulse chase experiments, the bulk of cell-associated Ab-sFv remained in an endo H-sensitive form during a period when substantial amounts of sFv were accumulating in the medium in an endo H-resistant form.

This indicates that most of the cell-associated sFv had not yet passed through the medial Golgi, where carbohydrate modifications conferring endo H resistance occur (Dunphy, W.G. and Rothman, J.E. Cell 42:13-21 (1985); Tarentino, A.L., et al. J. Biol. Chem. 249:818-824 (1974)). Thus the exit of Ab-sFv from the ER appears to be the rate limiting step in their secretion.

As a result of the work presented herein, soluble, secretable sFv molecules, including sFv fusion molecules, can be produced in mammalian cells. All secreted antibody sFv specifically bound the appropriate antigen, and where tested, at least 90% of the secreted sFv was functional. Therefore, mammalian cells can properly fold and secrete antibody sFv and the presence of oligosaccharide can enhance their rates of secretion. These sFv molecules are biologically active and readily isolated and purified from cell culture medium.

The sFv molecules produced by the method described herein are useful in any procedure where intact immunoglobulins (IgG), fragmented IgG, analogous Ig superfamily sFv analogues, or chimeric derivatives are used. An sFv antibody produced by the method described herein can be used in a diagnostic immunoassay procedure to detect the presence of a specific protein which is indicative of a disease condition. For example, an sFv antibody specific for a tumor marker found in blood or urine can be used in an ELISA to screen patients for a particular type of cancer. As another example, an sFv receptor protein can be used in an assay to screen peptides for biological activity which enhance or inhibit receptor activity. In particular, due to their smaller size, these sFv molecules are useful as in vivo targeting agents. For example, the sFv molecule can be used to deliver effector molecules such as cellular toxins to targeted cells or to deliver radioisotope to tumors

(Huston, J.S., et al., Intern Rev. Immurol., 10:195-219 (1993)).

The expression of sFv molecules in mammalian cells, as described herein, allows a rapid determination of the biological activity of a particular construct. This could be especially useful in the production and testing of fusion proteins, where both the sFv and its fusion partner must be correctly folded. In many such cases, it may prove impossible to fold such constructs in vitro, while the folding machinery in the ER of mammalian cells can produce active sFv fusion proteins in vivo. Additionally, the method described herein provides a rapid means of screening numerous sFv constructs, or provides the means to determine if a particular sFv construct encodes an active sFv protein (e.g., on a pilot scale). Once it has been determined that an active sFv protein is produced, other systems, such as stably transfected cell lines or transgenic animals or plants, can be used for large-scale production of the protein.

The present invention will now be illustrated by the following examples, which will further and more specifically illustrate the invention.

Example 1: sFv Constructs

Constructs and primers used are shown in Figure 1. All antibody sFv constructs contain a V_L-linker-V_H, L-chain leader sequences, and a C-terminal peptide tag (from the c-myc proto-oncogene). The TcR sFv construct contained a leader sequence to direct protein to the ER. The following primers were used to produce the constructs:

Primer 1, tgttaactgctcact TCT AGA ATG AGG ACC CCT GCT CAG TTT CTT GGA ATC TTG TTG CTC TGG TTT CCA GGT ATC AAA TGT GAC ATC AAG ATG ACC CAG TCT. (SEQ ID NO: 1)

Primer 2, atata GAA TTC CTC GAG GAG CTC TTA TTA ATT CAG ATC CTC TTC TGA GAT GAG TTT TTG TTC TGA TAA AGC TTT TGA GGA GAC TGT. (SEQ ID NO: 2)

Primer 3, atata GAA TTC CTC GAG GAG CTC TTA TTA GAG TTC GTC CTT TTC GCT ATT CAG ATC CTC TTC TGA GAT GAG TTT TTG TTC TGA TAA AGC TTT TGA GGA GAC TGT. (SEQ ID NO: 3)

Primer 4, tgttaactgctcact TCT AGA ATG AGG ACC CCT GCT CAG TTT CTT GGA ATC TTG TTG CTC TGG TTT CCA GGT ATC AAA TGT GAC GTC GTC ATG ACC CAG TCT CCA GCA. (SEQ ID NO: 4)

Primer 5, atata GGA TCC ATG AGG GCC CCT ACT GTC. (SEQ ID NO: 5)

Primer 6, atata GCG GCC GCC ACT CCC ACC TCC GCC AGA ACC TCC GCC TCC TGA TCC GCC ACC TCC TTT GAT TTC CAG CTT GGT GCC. (SEQ ID NO: 6)

Primer 7, atata GGC GGC CGC GAG GTG CAG CTG GTG GAG. (SEQ ID NO: 7)

Primer 8, atata CTC GAG TTA TTA ATT CAG ATC CTC TTC TGA GAT GAG TTT TTG TTC TGA TGA GGA GAC GGT GAC CAT GGT. (SEQ ID NO: 8)

Primer 9, atata TCT AGA GAG AAG ACA ACC AGC GAT TGG ACA GGG GCC ATG CAG AGG AAC CTG GGA GCT GTG CTG GGG ATT CTG TGG GTG CAG ATT TGC TGG GTG AGA GGA GAT CAG GTG GAG CAG AGT CCT TCA GCC. (SEQ ID NO: 9)

Primer 10, atata GGA TCC TCA CTA AGT CAC ATT TCT CAG ATC CTC. (SEQ ID NO: 10)

In all primers, underlined sequences indicate restriction sites, lower case letters designate nucleotides added to facilitate cutting with restriction enzymes, and bold letters designate added sequences encoding for amino acid residues not present in the template.

U7.6 and OKT9 AbsFv proteins and the 2B4 TcR-sFv constructs have been described in (Kurucz, I., et al. Proc. Natl. Acad. Sci. U.S.A. 90:3830-3834 (1993);

Nicholls, P.J., et al. J. Immunol. Methods 165:81-91 (1993)). The nucleic acid sequence of U7.6 (SEQ ID NOs: 14 and 16) are shown in Figure 2A and 2B (V_H region and V_L region, respectively). Plasmids containing these constructs were used as polymerase chain reaction (PCR) templates. The OKT9 sFv construct was amplified using sense primer 1 that introduced an Xba I site and a light chain leader sequence and anti-sense primer 2 that introduced a c-myc peptide sequence, two stop codons, and a Sac I site. To construct the OKT9+KDEL sFv sense primer 1 and an anti-sense primer 3 that is identical to primer 2 but adds an additional SEKDEL sequence at the C terminal end were used. The PCR products were directly ligated into PCRIOOO (Invitrogen, San Diego, CA), according to manufacturers instructions, and subcloned into the Xba I and Sac I site of pSVL (Pharmacia, Piscataway, NJ). The U7.6 sFv construct was amplified using sense PCR-primer 4 that introduced an Xba I site and a light chain leader sequence followed by an Aat II site, and anti-sense primer 2.

Following the digestion of the purified PCR product with Xba I and Sac I the construct was directly ligated into the Xba I and Sac I site of pSVL. The 2C11 sFv was constructed from cDNA using primers based on the 2C11 V region sequences, kindly supplied by Dr. J. Yun Tso (Protein Design Labs, Mountain View, CA). Specifically primed first strand cDNA was synthesized from mRNA isolated from 2C11 cells (FastTrack Invitrogen, San Diego, CA) by primer extension using reverse transcriptase (Superscript, Gibco/BRL, Grand Island, NY). The light chain cDNA was synthesized using anti-sense primer 6 that introduces a $(G_4S)_3$ linker and a Not I site. This cDNA was used for amplification of V_L using anti-sense primer 6 and sense primer 5, that introduces a BamH I site. The heavy chain cDNA was synthesized using

anti-sense primer 8 that introduces a myc-peptide sequence and a Xho I site. This cDNA was used for amplification of the light chain using anti-sense primer 8 and sense primer 7 that introduces a Not I site at the 5' end. The PCR products were purified, blunted using T4 DNA polymerase (Boehringer Mannheim Indianapolis, IN) phosphorylated with T4 polynucleotide kinase (Gibco/BRL, Grand Island, NY) and sub-cloned into pcDNA/AMP (Invitrogen, San Diego, CA) that had been cut with EcoR V and treated with calf alkaline phosphatase (Boehringer Mannheim). The light chain was excised with BamH I and Not I and the heavy chain with Not I and Xho I and both chains were simultaneously ligated into pcDNA/AMP digested with BamH I and Xho I.

Cloned 2B4 TcR-sFv (Kurucz, I., et al. Proc. Natl. Acad. Sci. U.S.A. 90:3830-3834 (1993)) was used as the PCR template for the TcR-sFv construct. The 2B4 sFv construct was amplified using sense primer 9 introducing an Xba I site (underlined), 30 bp of the α-chain 5' non-coding region and the α-chain leader sequence and anti-sense primer 10 that introduced a BamH I site. The PCR products were directly ligated into PCRIOOO and subcloned into the Xba I and BamH I sites of pSVL.

Site directed mutagenesis of U7.6

Mutations in the U7.6 sFv constructs were introduced using a Transformer Mutagenesis Kit (Clonetech Laboratories Inc, Palo Alto, CA) according to the manufacturer's instructions except that ten times more plasmid was used than recommended. Colonies were screened using restriction enzyme digestion and plasmids from mutant clones were sequenced with a Sequenase Version 2.0 Kit (United States Biochemical, Cleveland, OH). Constructs were recloned to eliminate possible changes introduced into the vector during mutagenesis.

The primers used were: U7.6 -CYS, C TGC CAG CAG TaC AGT GGT TAC CCG, (SEQ ID NO: 11) introduced a tyrosine in place of cysteine 91 of V_L (the original U7.6 V_L clone contained a tyrosine residue at position 91; cysteine 91 was inadvertently introduced at a subsequent recloning step, probably as a PCR-induced mutation); U7.6+ASN, GGC GCT TCA GTG AAt ATA TCC TGC AAG GC, (SEQ ID NO: 12) introduced an asparagine for lysine 19 ofV_H. As a selection primer we used CCC TTT CGT CTT CAA Gtt TTC TCA TGT TTG ACA GC (SEQ ID NO: 13) which removed an EcoR I site from the vector. In the above primers, lower case letters designate the mutated nucleotides. The double mutant, U7.6 -CYS+ASN, was produced by using all three primers simultaneously.

Example 2: Transfection and Metabolic Labeling

Cell Lines and Antibodies

The following cell lines and monoclonal antibodies were used: COS-7 monkey kidney fibroblasts and K562 human erythroleukemia cells (American Type Culture Collection, Rockville, MD), 2B4 murine T hybridoma cells (Hedrick, S.M., et al., Cell, 30:141-152 (1982)), 145-2C11 hybridoma cells and mAb against murine CD3 ε chain (Leo, O., et al., Proc. Natl. Acad. Sci. USA 84:1374-1378 (1987)), OKT9 mAb against human transferrin receptor (TfR) (Goding, J.W. and Burns, G.F. J. Immunol. 127:1256-1258 (1981); Schneider, C., et al., J. Biol. Chem. 257:8516-8522 (1982)), W6/32 mAb against human MHC Class I molecules (Barnstable, C.J., et al., Cell, 14:9-20 (1978)), H57 mAb against the C_β domain of murine TcRs (Kubo, R.T., et al., J. Immunol., 142:2736-2742 (1989)), 9EIO mAb against a c-myc peptide (Evan, G.I., et al., Mol. Cell Biol., 5:3610-3616 (1985)), and A2B4 mAb, which is specific for the 2B4 TcR (Samelson, L.E., et al., Proc. Natl. Acad. Sci. U.S.A. 80:6972-6976 (1983)).

Polyclonal rabbit anti-mouse IgG was from Cappel (Organon Technika, Durham, NC). COS-7 cells cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (100 units/ml), streptomycin (100 units/ml), 2 mM L-glutamine, (all from Biofluids, Rockville, MD), and 10% fetal calf serum (Hazelton Biochemicals, Lenexia, KS) were plated in 10-cm culture dishes. The next day the medium was changed and two hours later the cells were transfected with 20 μg of plasmid DNA [produced in E. coli strain HBIO1 (Gibco/BRL) and purified with a Qiagen (Chatsworth, CA) Plasmid Kit] using the calcium phosphate precipitation method (Davis, L.G., et al., Basic methods in molecular biology, Elsevier, New York (1986)).

For pulse chase experiments COS-7 cells were trypsinized 16 h after transfection and replated in three 10-cm culture dishes to generate a uniform population of cells and minimize dish to dish variation. The cells were then allowed to grow for an additional 24 h. Cells in a 10-cm dish that were 90% confluent were preincubated for 20 min at 37° C in methionine free-DMEM containing 10% fetal calf serum (dialyzed against PBS) after which [^{35}S]methionine (Trans^{35}S-label [ICN Radiochemicals, Irvine, CA]) was added to a concentration of O. 15 mCi/ml. The cells were pulsed for 1 h at 37° C and then washed and chased in DMEM containing 15 mg/ml of L-methionine (chase medium)(Sigma Chemical Co., St. Louis, MO). Cells and culture media were collected separately. In some pulse-chase experiments cells were pretreated for 2 h, pulsed for 1 h, and chased for 2 h in medium that contained 3/μg/ml of tunicamycin (Sigma) throughout the experiment.

Example 3: Isolation and Purification of Proteins

Immunoprecipitation and endoglycosidase treatment

Culture media were used immediately for immunoprecipitation. Cells were washed 3 times with cold PBS, scraped from the dishes and lysed overnight in 250 µl lysis buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCI, 0.5% NP-40 (Calbiochem, San Diego, CA), 1mM phenyl methyl sulfonyl fluoride (Sigma), pH 8.0). Nuclei were pelleted by centrifugation for 30 min at 12,000 g. Culture media (3 or 6 ml) or cell lysates (250 µl) were incubated for 3 h at 4° C with gentle tumbling with 20 µl of packed Protein A-Sepharose beads (Pharmacia) precoated with rabbit IgG. The precleared samples were then centrifuged and incubated with 3 µg of either 9EIO or A2B4 mAb for 3 h, followed by 3-16 h incubation at 4° C with 20-30 µl of packed Protein A-Sepharose precoated with rabbit anti-mouse Ig. The immunoprecipitates were washed three times with 5% sucrose, 1% NP40, 0.5 M NaCI, 50 mM Tris, 5 mM EDTA, pH 7.2 and once with 50 mM Tris, 150 mM NaCI, 1% Triton X-100 (Sigma), 0.15% SDS, 1% sodium deoxycholate, and solubilized in 40 µl reducing SDS polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. Sequential immunoprecipitations using the same antibody were performed until less than 10% of the original protein remained in the supernatant. In some experiments immunoprecipitates were digested with endoglycosidase H (endo H) (Genzyme, Cambridge, MA), as described in (Kearse, K.P. and Singer, A., J. Immunol. Methods (1994)). Following endo H digestion, an equal volume of reducing SDS-PAGE sample buffer was added.

SDS-PAGE, autoradiography and densitometry

SDS-PAGE was performed using a Pharmacia PhastSystem with 12.5% homogeneous Phastgels. Samples were heated to 95° C for 5 min, beads were removed by centrifugation and

3.5 μl aliquots of supernatant were applied to the gel. After electrophoresis, gels were incubated with shaking twice for 20 min in dimethyl sulfoxide, once for 2 h in 20% w/v 2,5-diphenyloxazole in dimethyl sulfoxide, and once for 30 min in H_2O. Gels were dried in a microwave oven for 20 min at the lowest power level and exposed to film (Kodak X-OMAT AR, Rochester, NY) at -70° C for 1-3 days. Autoradiograms were quantified with a Molecular Dynamics Computing Densitometer using Imagequant soilware. Exposure times were chosen to ensure linearity between radioactivity and band density. Multiple loadings of identical samples gave standard deviations in band densities of less than 10%.

Example 4: sFv Binding Studies

Binding of sFv proteins was tested using the radiolabeled material present in the media of transfected COS-7 cells after either a 2 h or a 6 h chase. Binding of OKT9 and 2C11 sFv media was tested on K562 (TfR^+) and 2B4 ($CD3^+$) cells, respectively. Chase media containing (4 ml) 10 mM HEPES (Biofluids) were incubated for 4 hr at with 5-10 x 10^6 cells in 6 well plates, in the presence or absence of excess inhibiting or control antibodies. Inhibiting antibodies were the parental mAbs from which the sFv proteins were derived, and control antibodies were W6/32 and H57 for OKT9 and 2C11 respectively, both of which bind to the same cells as the sFv proteins, but to different antigens. After incubation and washing, cells were lysed in 250 μl lysis buffer, and sFv proteins were immunoprecipitated and analyzed by SDS-PAGE as described above. U7.6 sFv was tested for binding by incubation with gentle tumbling of 1.5 or 4ml of chase medium with 50 μl of packed DNP-Sepharose beads for 16 h in the absence or presence of 1 mM DNP-ε-aminocaproate. The beads were washed and the bound sFv was solubilized

in 40 μl reducing sample buffer in preparation for SDS-PAGE. In some studies, U7.6 sFv supernatants were incubated a second time with 50 μl of DNP-beads. The doubly-depleted supernatants were then assayed for residual sFv by immunoprecipitating with 9E 10 and rabbit anti-mouse protein A beads. Fractions were then analyzed using SDS-PAGE and the bands were quantified by densitometry.

In order to measure relative affinities of the U7.6 mutant sFv constructs, 1 ml aliquots of ^{35}S-methionine labeled chase media containing .01% sodium azide, 10 mM HEPES, and graded amounts of DNP-ε-aminocaproate were incubated overnight at 4°C with 40 μl of packed DNP-Sepharose beads. The beads were washed, and bound sFv removed by addition of 1 mM DNP-hapten for 6 hr at 4°C. The eluted sFv was immunoprecipitated with 9E10 mAb and protein A-Sepharose, and the beads were washed, divided into 4 equal portions, and heated for 5 min at 94°C in 100 μl of elution buffer (20mM Tris, 1mM EDTA, 2% SDS and 5% 2ME pH 7.8). The eluate (85μl) was transferred directly to 2.5ml of Ecolume (ICN Cleveland Ohio) scintillation solution ad counted in a liquid scintillation counter.

Example 5: Dissociation of OKT9-sFv from K562 Cells

To measure the dissociation rate of COS-7 cell-produced OKT9-sFv from K562 cells, tubes containing 6x10^6 K562 or 2B4 (control) cells and 1 ml of COS-7 medium containing ^{35}S-methionine labeled OKT9-sFv and supplemented with .01% sodium azide, 10 mM HEPES were incubated 1 h at 4°C. Cells were centrifuged for 5 min at 425xG. To determine the maximal amount of sFv bound (0 time), cells were resuspended in Hank's balanced salt solution without phenol red containing 0.1% BSA and .01% sodium azide (wash buffer), immediately spun for 10 sec

in an Eppendorf microcentrifuge, and the pellet frozen. Other samples were suspended in 1 ml wash buffer containing 50 μg OKT9 mAb, incubated for various times at 4°C, spun for 10 sec, and pellets frozen. Cell pellets were lysed and sFv immunoprecipitated with 9E10 mAb and protein A-Sepharose beads, and taken for scintillation counting as described above. Quadruplicate samples were averaged, and the amount of radioactivity associated with 2B4 cells was considered background and was subtracted from the amount binding to K562 cells.

The dissociation rate was also measured using bacterially-produced OKT9-sFv and flow cytometry. OKT9-sFv was produced and refolded from bacterial inclusion bodies, and labeled with fluorescein isothiocyante (FITC) as described in Segal, D.M. et al., Meth. Enzymol, 150:478-492 (1987) K562 cells (10^7) were incubated for 1 h at 4°C with 2 μg OKT9-sFv-FITC, with or without 50 μg OKT9 mAb in a total volume of 1 ml. Cells were centrifuged for 5 min at 425xG, resuspended in 1 ml wash buffer containing 50 μg OKT9 mAb, and incubated at 4°C. At various times, samples were analyzed by flow cytometry. Mean fluorescence values were determined and control values (mean fluorescence of samples incubated with FITC-sFv in the presence of excess unlabeled mAb) were subtracted at each time point.

Equivalents

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims:

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: The United States of America, as represented by the Secretary of the Health and Human Services
(B) STREET: 6011 Executive Boulevard, Susite 325
(C) CITY: Rockville
(D) STATE/PROVINCE: Maryland
(E) COUNTRY: U.S.A.
(F) POSTAL CODE/ZIP: 20852-3804

(i) APPLICANT:
(A) NAME: Creative BioMolecules, Inc.
(B) STREET: 35 South Street
(C) CITY: Hopkinton
(D) STATE/PROVINCE: Massachusetts
(E) COUNTRY: U.S.A.
(F) POSTAL CODE/ZIP: 01748

(ii) TITLE OF INVENTION: Method of Producing Single-Chain Fv Molecules

(iii) NUMBER OF SEQUENCES: 17

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Hamilton, Brook, Smith & Reynolds, P.C.
(B) STREET: Two Militia Drive
(C) CITY: Lexington
(D) STATE: Massachusetts
(E) COUNTRY: U.S.
(F) ZIP: 02173

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/292,124
(B) FILING DATE: 17-AUG-1994

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Brook, David E.
(B) REGISTRATION NUMBER: 22,592
(C) REFERENCE/DOCKET NUMBER: CBM94-01

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 617-861-6240
(B) TELEFAX: 617-861-9540

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 102 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(ii) MOLECULE TYPE: synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TGTTAACTGC TCACTTCTAG AATGAGGACC CCTGCTCAGT TTCTTGGAAT CTTGTTGCTC 60

TGGTTTCCAG GTATCAAATG TGACATCAAG ATGACCCAGT CT 102

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 86 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(ii) MOLECULE TYPE: synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATATAGAATT CCTCGAGGAG CTCTTATTAA TTCAGATCCT CTTCTGAGAT GAGTTTTTGT 60

TCTGATAAAG CTTTTGAGGA GACTGT 86

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 104 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(ii) MOLECULE TYPE: synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATATAGAATT CCTCGAGGAG CTCTTATTAG AGTTCGTCCT TTTCGCTATT CAGATCCTCT 60

TCTGAGATGA GTTTTTGTTC TGATAAAGCT TTTGAGGAGA CTGT 104

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 108 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(ii) MOLECULE TYPE: synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TGTTAACTGC TCACTTCTAG AATGAGGACC CCTGCTCAGT TTCTTGGAAT CTTGTTGCTC 60

TGGTTTCCAG GTATCAAATG TGACGTCGTC ATGACCCAGT CTCCAGCA 108

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(ii) MOLECULE TYPE: synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ATATAGGATC CATGAGGGCC CCTACTGTC 29

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 80 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(ii) MOLECULE TYPE: synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ATATAGCGGC CGCCACTCCC ACCTCCGCCA GAACCTCCGC CTCCTGATCC GCCACCTCCT 60

TTGATTTCCA GCTTGGTGCC 80

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(ii) MOLECULE TYPE: synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATATAGGCGG CCGCGAGGTG CAGCTGGTGG AG 32

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 74 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(ii) MOLECULE TYPE: synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ATATACTCGA GTTATTAATT CAGATCCTCT TCTGAGATGA GTTTTTGTTC TGATGAGGAG 60

ACGGTGACCA TGGT 74

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 131 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(ii) MOLECULE TYPE: synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATATATCTAG AGAGAAGACA ACCAGCGATT GGACAGGGGC CATGCAGAGG AACCTGGGAG 60

CTGTGCTGGG GATTCTGTGG GTGCAGATTT GCTGGGTGAG AGGAGATCAG GTGGAGCAGA 120

GTCCTTCAGC C 131

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(ii) MOLECULE TYPE: synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ATATAGGATC CTCACTAAGT CACATTTCTC AGATCCTC 38

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(ii) MOLECULE TYPE: synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CTGCCAGCA GTACAGTGGT TACCCG 26

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(ii) MOLECULE TYPE: synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GGCGCTTCAG TGAATATATC CTGCAAGGC 29

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(ii) MOLECULE TYPE: synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CCCTTTCGTC TTCAAGTTTT CTCATGTTTG ACAGC 35

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 360 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..360

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

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CAG GTC CAA CTG CAG CAG TCT GGA CCT GAG CTG GAG AAG CCT GGC GCT        48
Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Glu Lys Pro Gly Ala
 1               5                  10                  15

TCA GTG AAG ATA TCC TGC AAG GCT TCT GGT TAC TCA TTC ACT GGC TAC        96
Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ser Phe Thr Gly Tyr
            20                  25                  30

ATC ATG AAC TGG GTA AAA CAG AAC AAT GGA AAG AGC CTT GAG TGG ATT       144
Ile Met Asn Trp Val Lys Gln Asn Asn Gly Lys Ser Leu Glu Trp Ile
        35                  40                  45

GGA AAT ATT GCT CCT TAC TAT GGT GGT ACT AGC TAC AAC CAG AAG TTC       192
Gly Asn Ile Ala Pro Tyr Tyr Gly Gly Thr Ser Tyr Asn Gln Lys Phe
    50                  55                  60
```

```
AAG GGC AAG GCC ACA TTG ACT GTA GAC AAA TCC TCC AGC ACA GCC TAC        240
Lys Gly Lys Ala Thr Leu Thr Val Asp Lys Ser Ser Ser Thr Ala Tyr
 65                  70                  75                  80

ATG CAG CTA AGC AGC CTG ACA TCT GAG GAC TCT GCA GTC TAT TTC TGT        288
Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys
                85                  90                  95

GCA AGA TGG GGA GGT ACT ATG ATT ACG GGT CTT GAC TAC TGG GGC CAA        336
Ala Arg Trp Gly Gly Thr Met Ile Thr Gly Leu Asp Tyr Trp Gly Gln
            100                 105                 110

GGC ACC ACT CTC ACA GTC TCC TCA                                       360
Gly Thr Thr Leu Thr Val Ser Ser
        115                 120
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(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 120 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

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Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Glu Lys Pro Gly Ala
  1               5                  10                  15

Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ser Phe Thr Gly Tyr
             20                  25                  30

Ile Met Asn Trp Val Lys Gln Asn Asn Gly Lys Ser Leu Glu Trp Ile
         35                  40                  45

Gly Asn Ile Ala Pro Tyr Tyr Gly Gly Thr Ser Tyr Asn Gln Lys Phe
     50                  55                  60

Lys Gly Lys Ala Thr Leu Thr Val Asp Lys Ser Ser Ser Thr Ala Tyr
 65                  70                  75                  80

Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys
                85                  90                  95

Ala Arg Trp Gly Gly Thr Met Ile Thr Gly Leu Asp Tyr Trp Gly Gln
            100                 105                 110

Gly Thr Thr Leu Thr Val Ser Ser
        115                 120
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(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 327 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..327

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

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GAT ATT GTC ATG ACC CAG TCT CCA GCA ATC ATG TCT GCA TCT CCA GGG          48
Asp Ile Val Met Thr Gln Ser Pro Ala Ile Met Ser Ala Ser Pro Gly
 1               5                  10                  15

GAA AAG GTC ACC ATG ACC TGC AGG GCC AGC TCA AGT GTA AGT TCC ACT          96
Glu Lys Val Thr Met Thr Cys Arg Ala Ser Ser Ser Val Ser Ser Thr
            20                  25                  30

TAC TTC CAC TGG TAC CAG CAG AAG TCA GGT GCC TCC CCC AAA CTC TGG         144
Tyr Phe His Trp Tyr Gln Gln Lys Ser Gly Ala Ser Pro Lys Leu Trp
        35                  40                  45

ATT TAT AGC ACA TCC ACC TTG GCT TCT GGA GTC CCT GCT CGC TTC AGT         192
Ile Tyr Ser Thr Ser Thr Leu Ala Ser Gly Val Pro Ala Arg Phe Ser
    50                  55                  60

GGC AGT GGG TCT GGG ACC TCT TAC TCT CTC ACA ATC AGC AGT GTG GAG         240
Gly Ser Gly Ser Gly Thr Ser Tyr Ser Leu Thr Ile Ser Ser Val Glu
65                  70                  75                  80

GCT GAA GAT GCT GCC ACT TAT TAC TGC CAG CAG TAC AGT GGT TAC CCG         288
Ala Glu Asp Ala Ala Thr Tyr Tyr Cys Gln Gln Tyr Ser Gly Tyr Pro
                85                  90                  95

CTC ACG TTC GGT GCT GGG ACC AAG CTG GAG CTG AAA CGC                     327
Leu Thr Phe Gly Ala Gly Thr Lys Leu Glu Leu Lys Arg
            100                 105
```

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 109 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

```
Asp Ile Val Met Thr Gln Ser Pro Ala Ile Met Ser Ala Ser Pro Gly
 1               5                  10                  15

Glu Lys Val Thr Met Thr Cys Arg Ala Ser Ser Ser Val Ser Ser Thr
            20                  25                  30

Tyr Phe His Trp Tyr Gln Gln Lys Ser Gly Ala Ser Pro Lys Leu Trp
        35                  40                  45

Ile Tyr Ser Thr Ser Thr Leu Ala Ser Gly Val Pro Ala Arg Phe Ser
    50                  55                  60

Gly Ser Gly Ser Gly Thr Ser Tyr Ser Leu Thr Ile Ser Ser Val Glu
65                  70                  75                  80
```

Ala Glu Asp Ala Ala Thr Tyr Tyr Cys Gln Gln Tyr Ser Gly Tyr Pro
85 90 95

Leu Thr Phe Gly Ala Gly Thr Lys Leu Glu Leu Lys Arg
100 105

CLAIMS

The invention claimed is:

1. A method of producing single-chain Fv molecules in eukaryotic cells comprising:
   (a) modifying a nucleic acid sequence which encodes a single-chain Fv molecule to include at least one non-naturally occurring glycosylation site in the nucleic acid sequence, thereby producing a single-chain Fv construct;
   (b) introducing the single-chain Fv construct of step (a) into a vector capable of expressing said construct in a eukaryotic cell and transfecting said vector containing the single-chain Fv construct into a eukaryotic cell; and
   (c) maintaining said cell transfected with the vector of step (b) in cell culture medium under conditions sufficient for expression of the single-chain Fv construct within the cell and secretion of the expressed single-chain Fv protein product from the cell into the cell culture medium, thereby producing a single-chain Fv molecule.

2. A method of producing single-chain Fv molecules in mammalian cells comprising:
   (a) modifying a nucleic acid sequence which encodes a single-chain Fv molecule to include at least one non-naturally occurring glycosylation site in the nucleic acid sequence thereby providing a single-chain Fv construct;
   (b) introducing the single-chain Fv construct of step (a) into a vector capable of expressing said construct in a mammalian cell and transfecting said vector into a mammalian cell; and

(c) maintaining said cell transfected with the vector of step (b) in cell culture medium under conditions sufficient for expression of the single-chain Fv construct within the cell and secretion of the expressed single-chain Fv protein product from the cell into the cell culture medium, thereby producing a single-chain Fv molecule.

3. The method of Claim 2 wherein the mammalian cell is selected from the group consisting of: COS-7 monkey kidney fibroblast cells; K562 human erythroleukemia cells; 293 cells; myeloma cells; and Chinese hamster ovary cells.

4. The method of Claim 2 wherein the glycosylation site of step (a) is an N-linked glycosylation site.

5. The method of Claim 4 wherein the glycosylation site is encoded by the nucleic acid sequence Asn-X-Ser/Thr.

6. The method of Claim 2 wherein the nucleic acid sequence encodes single-chain Fv U7.6 (SEQ ID NOs: 14 and 16).

7. The method of Claim 6 wherein the modification to the U7.6 nucleic acid sequence to include at least one glycosylation site consists of an asparagine amino acid residue substituted for lysine 19 of V_H.

8. A single-chain Fv molecule produced by the method of Claim 2.

9. A method of increasing the rate of secretion of single-chain Fv molecules from mammalian cells comprising introducing into a single-chain Fv molecule at least one non-naturally occurring glycosylation site.

10. The method of Claim 9 wherein the glycosylation site is an N-linked glycosylation site.

11. The method of Claim 10 wherein the glycosylation site is encoded by the nucleic acid sequence Asn-X-Ser/Thr.

12. A method of decreasing intermolecular aggregation of single-chain Fv molecules in solution comprising introducing into a single-chain Fv molecule at least one non-naturally occurring glycosylation site.

13. The method of Claim 12 wherein the glycosylation site is an N-linked glycosylation site.

14. The method of Claim 13 wherein the glycosylation site is encoded by the nucleic acid sequence Asn-X-Ser/Thr.

15. A method of protecting a single-chain Fv molecule from proteolytic degradation comprising introducing into a single-chain Fv molecule at least one non-naturally occurring glycosylation site.

16. The method of Claim 15 wherein the glycosylation site is an N-linked glycosylation site.

17. The method of Claim 16 wherein the glycosylation site is encoded by the nucleic acid sequence Asn-X-Ser/Thr.

18. A method of decreasing the antigenicity of a single-chain Fv molecule comprising introducing into a single-chain Fv molecule at least one non-naturally occurring glycosylation site.

19. The method of Claim 18 wherein the glycosylation site is introduced into the linker sequence.

20. The method of Claim 18 wherein the glycosylation site is an N-linked glycosylation site.

21. The method of Claim 20 wherein the glycosylation site is encoded by the nucleic acid sequence Asn-X-Ser/Thr.

22. A method of modifying the ligand binding affinity of a single-chain Fv molecule comprising introducing into a single-chain Fv molecule at least one non-naturally occurring glycosylation site.

23. The method of Claim 22 wherein the glycosylation site is an N-linked glycosylation site.

24. The method of Claim 23 wherein the glycosylation site is encoded by the nucleic acid sequence Asn-X-Ser/Thr.

25. A method of modifying the pharmokenetics of a single-chain Fv molecule comprising introducing into a single-chain Fv at least one non-naturally occurring glycosylation site.

26. The method of Claim 25 wherein the glycosylation site is an N-linked glycosylation site.

27. The method of Claim 26 wherein the glycosylation site is encoded by the nucleic acid sequence Asn-X-Ser/Thr.

28. A method of screening single-chain Fv molecules for biological activity comprising:

(a) modifying a nucleic acid sequence which encodes a single-chain Fv molecule to include at least one non-naturally occurring glycosylation site in the nucleic acid sequence, thereby producing a single-chain Fv construct;

(b) introducing the single-chain Fv construct of step (a) into a vector capable of expressing said construct in a mammalian cell and transfecting said vector containing the single-chain Fv construct into a mammalian cell;

(c) maintaining said mammalian cell transfected with the vector of step (b) in cell culture medium under conditions sufficient for expression of the single-chain Fv construct within the cell and secretion of the expressed single-chain Fv protein product from the cell into the cell culture medium; and

(d) obtaining an aliquot of cell culture medium containing the single-chain Fv molecule and testing the aliquot for biological activity.

29. A secretable single-chain Fv molecule having one, or more, non-naturally occurring glycosylation site, or sites.

30. The single-chain Fv molecule of Claim 29 wherein the glycosylation site is an N-linked glycosylation site.

31. The single-chain molecule of Claim 29 wherein the single-chain Fv molecule is U7.6 and the glycosylation site of the U7.6 Fv molecule consists of an asparagine amino acid residue substituted for lysine 19 of V_H.

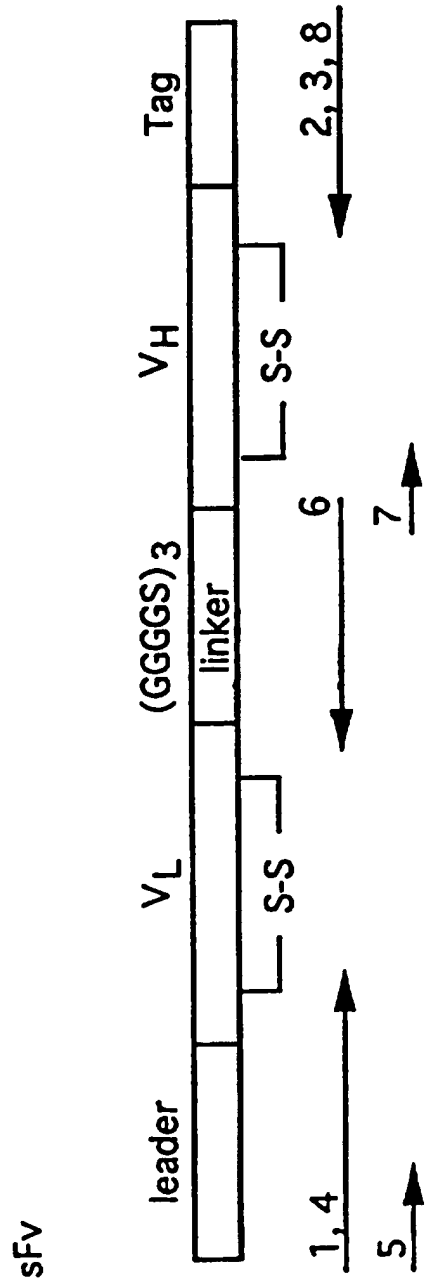


Figure 1

```
GAT ATT GTC ATG ACC CAG TCT CCA GCA ATC ATG TCT GCA TCT CCA GGG
Asp Ile Val Met Thr Gln Ser Pro Ala Ile Met Ser Ala Ser Pro Gly
  1               5                  10                  15

GAA AAG GTC ACC ATG ACC TGC AGG GCC AGC TCA AGT GTA AGT TCC ACT
Glu Lys Val Thr Met Thr Cys Arg Ala Ser Ser Ser Val Ser Ser Thr
            20                  25                  30

TAC TTC CAC TGG TAC CAG CAG AAG TCA GGT GCC TCC CCC AAA CTC TGG
Tyr Phe His Trp Tyr Gln Gln Lys Ser Gly Ala Ser Pro Lys Leu Trp
        35                  40                  45

ATT TAT AGC ACA TCC ACC TTG GCT TCT GGA GTC CCT GCT CGC TTC AGT
 Ile Tyr Ser Thr Ser Thr Leu Ala Ser Gly Val Pro Ala Arg Phe Ser
    50                  55                  60

GGC AGT GGG TCT GGG ACC TCT TAC TCT CTC ACA ATC AGC AGT GTG GAG
Gly Ser Gly Ser Gly Thr Ser Tyr Ser Leu Thr Ile Ser Ser Val Glu
 65                  70                  75                  80

GCT GAA GAT GCT GCC ACT TAT TAC TGC CAG CAG TAC AGT GGT TAC CCG
Ala Glu Asp Ala Ala Thr Tyr Tyr Cys Gln Gln Tyr Ser Gly Tyr Pro
                85                  90                  95

CTC ACG TTC GGT GCT GGG ACC AAG CTG GAG CTG AAA CGC
Leu Thr Phe Gly Ala Gly Thr Lys Leu Glu Leu Lys Arg
            100                 105
```

Figure 2A

```
CAG GTC CAA CTG CAG CAG TCT GGA CCT GAG CTG GAG AAG CCT GGC GCT
Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Glu Lys Pro Gly Ala
  1               5                  10                  15

TCA GTG AAG ATA TCC TGC AAG GCT TCT GGT TAC TCA TTC ACT GGC TAC
Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ser Phe Thr Gly Tyr
            20                  25                  30

ATC ATG AAC TGG GTA AAA CAG AAC AAT GGA AAG AGC CTT GAG TGG ATT
Ile Met Asn Trp Val Lys Gln Asn Asn Gly Lys Ser Leu Glu Trp Ile
        35                  40                  45

GGA AAT ATT GCT CCT TAC TAT GGT GGT ACT AGC TAC AAC CAG AAG TTC
Gly Asn Ile Ala Pro Tyr Tyr Gly Gly Thr Ser Tyr Asn Gln Lys Phe
    50                  55                  60

AAG GGC AAG GCC ACA TTG ACT GTA GAC AAA TCC TCC AGC ACA GCC TAC
Lys Gly Lys Ala Thr Leu Thr Val Asp Lys Ser Ser Ser Thr Ala Tyr
65                  70                  75                  80

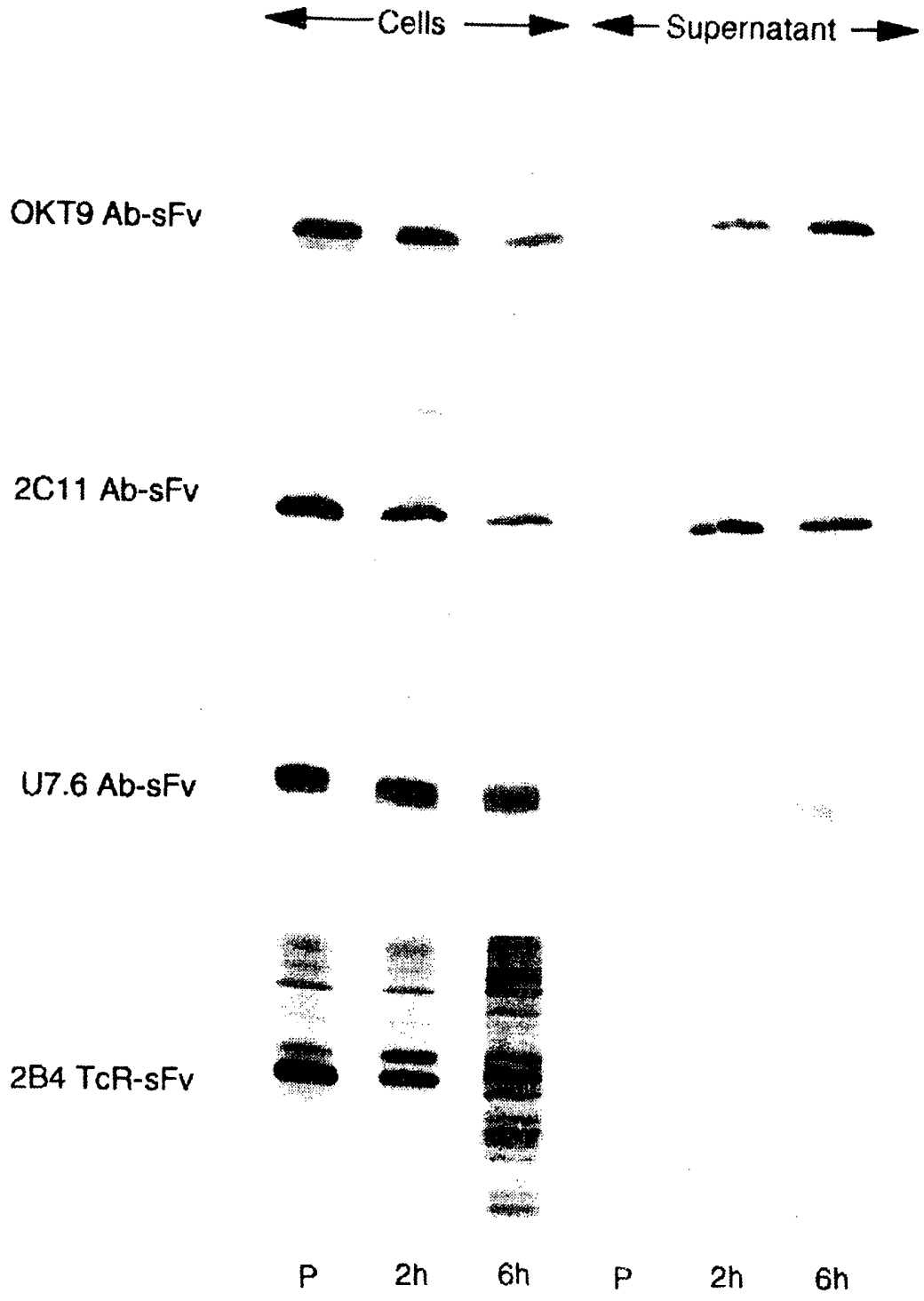
ATG CAG CTA AGC AGC CTG ACA TCT GAG GAC TCT GCA GTC TAT TTC TGT
Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys
                85                  90                  95

GCA AGA TGG GGA GGT ACT ATG ATT ACG GGT CTT GAC TAC TGG GGC CAA
Ala Arg Trp Gly Gly Thr Met Ile Thr Gly Leu Asp Tyr Trp Gly Gln
            100                 105                 110

GGC ACC ACT CTC ACA GTC TCC TCA
Gly Thr Thr Leu Thr Val Ser Ser
        115                 120
```

Figure 2B

FIG. 3A


Cells
Supernatant
OKT9 Ab-sFv
2C11 Ab-sFv
U7.6 Ab-sFv
2B4 TcR-sFv
P
2h
6h
P
2h
6h

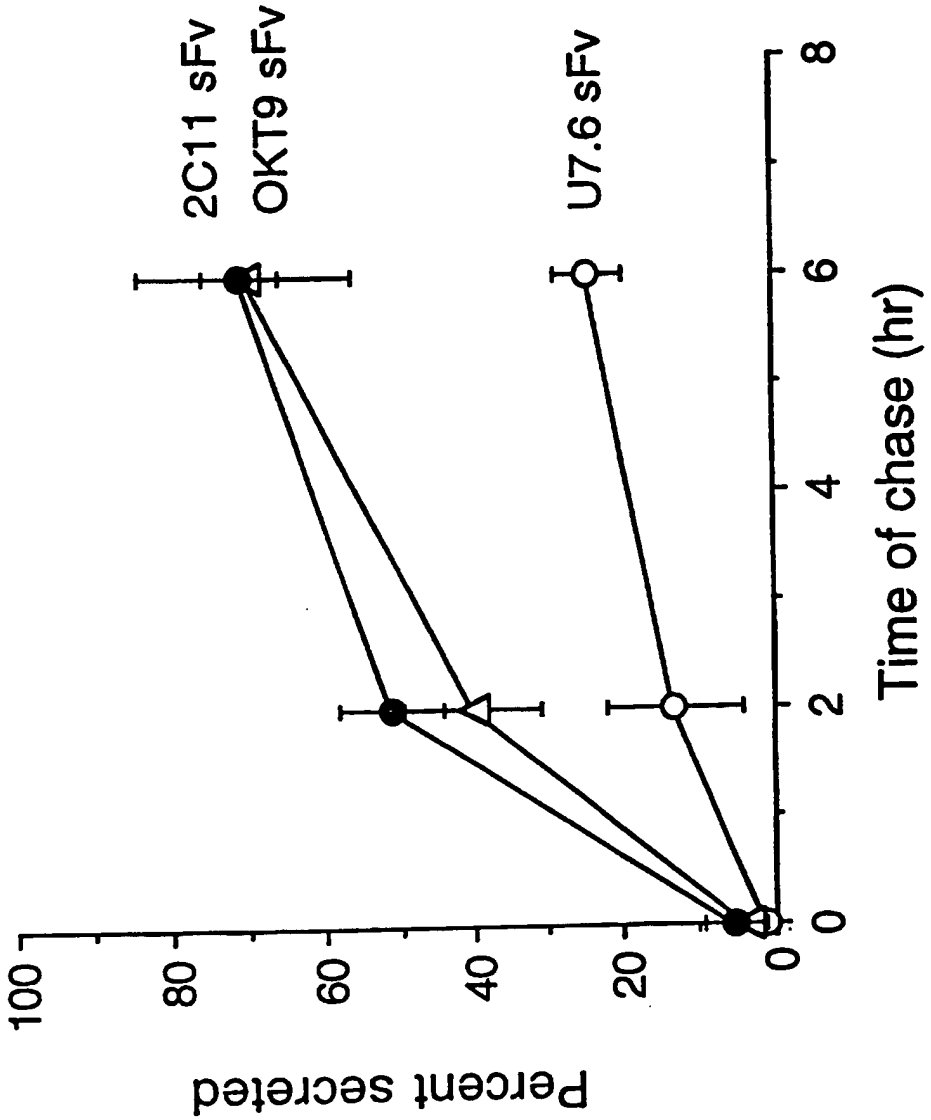

2C11 sFv
OKT9 sFv
U7.6 sFv
Percent secreted
Time of chase (hr)
0
20
40
60
80
100
0
2
4
6
8

Figure 3B

FIG. 4

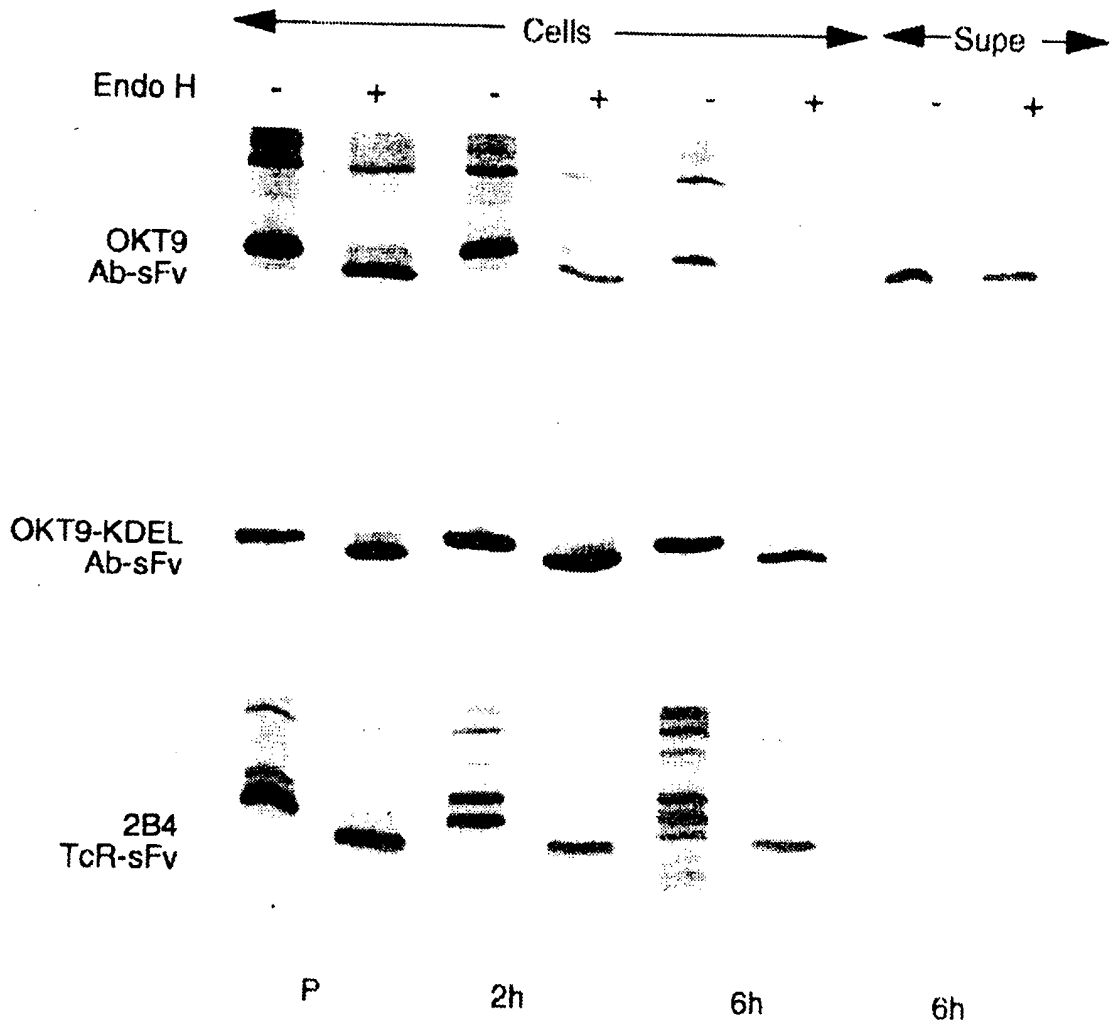
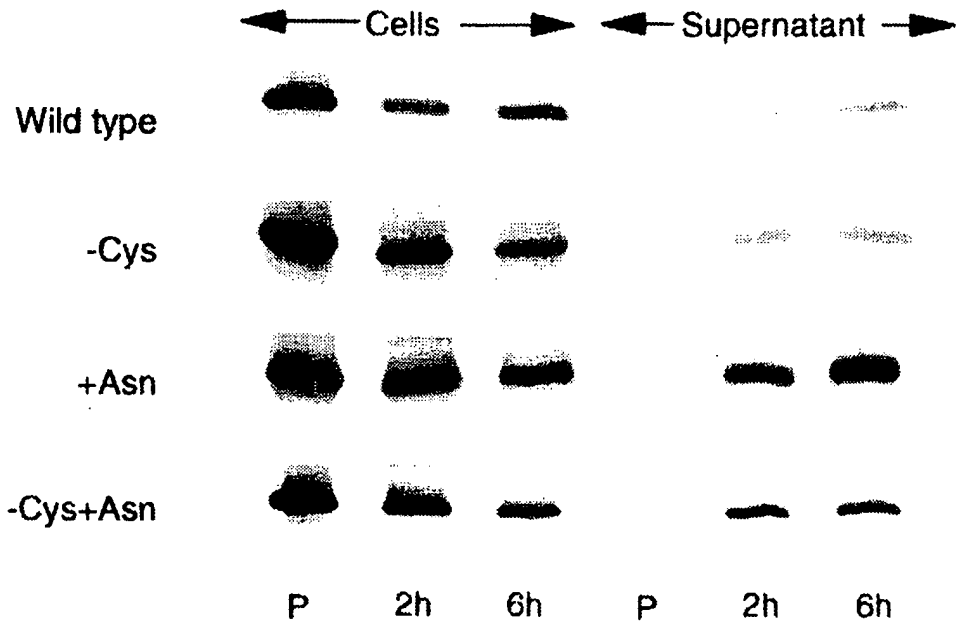

Cells
Supe
Endo H
- + - + - + - +
OKT9
Ab-sFv
OKT9-KDEL
Ab-sFv
2B4
TcR-sFv
P
2h
6h
6h

FIG. 5A



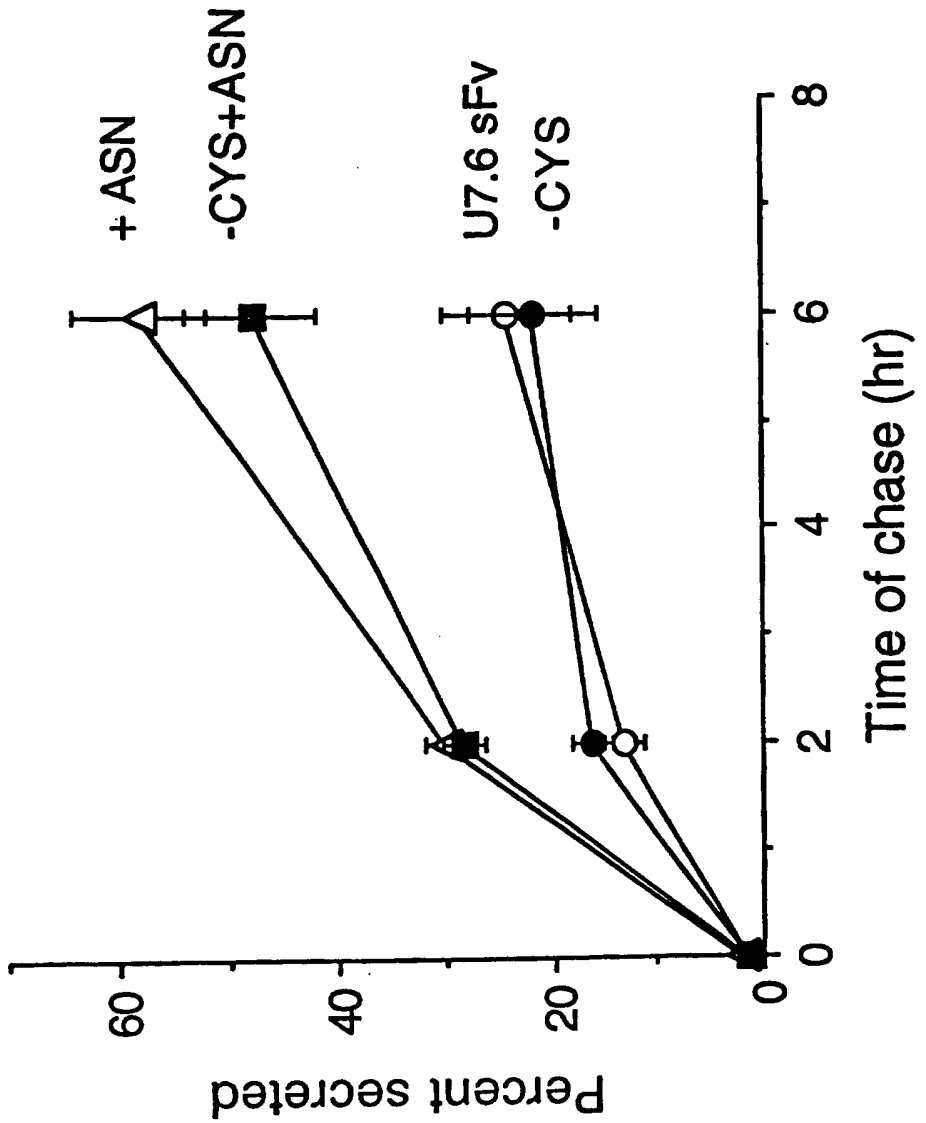

+ ASN
-CYS+ASN
U7.6 sFv
-CYS
Percent secreted
0
20
40
60
0
2
4
6
8
Time of chase (hr)

Figure 5B

FIG. 6

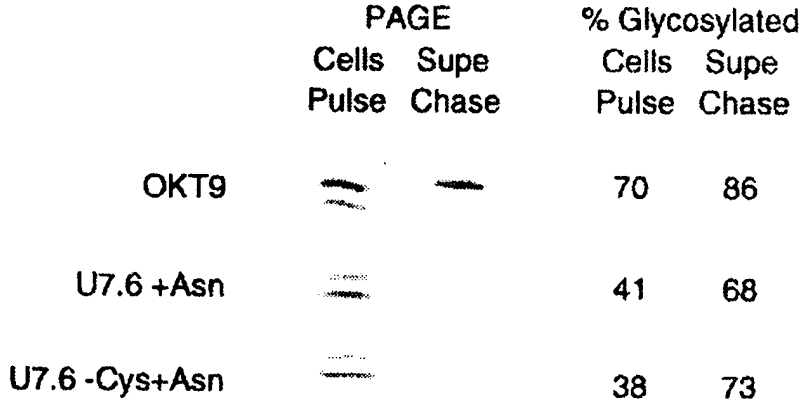
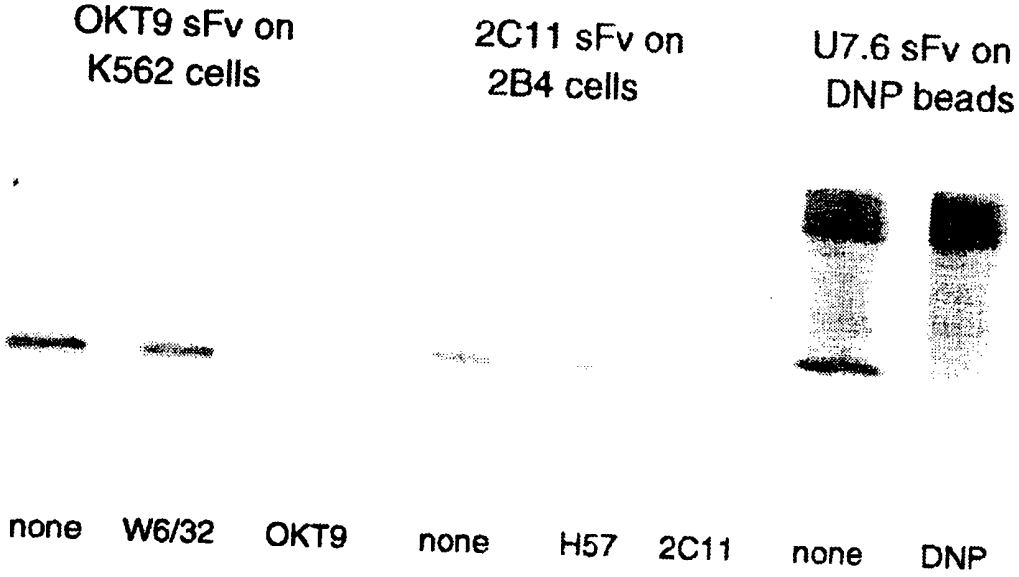

PAGE
Cells Pulse
Supe Chase
% Glycosylated
Cells Pulse
Supe Chase
OKT9
70
86
U7.6 +Asn
41
68
U7.6 -Cys+Asn
38
73

FIG. 7


OKT9 sFv on K562 cells
2C11 sFv on 2B4 cells
U7.6 sFv on DNP beads
none
W6/32
OKT9
none
H57
2C11
none
DNP

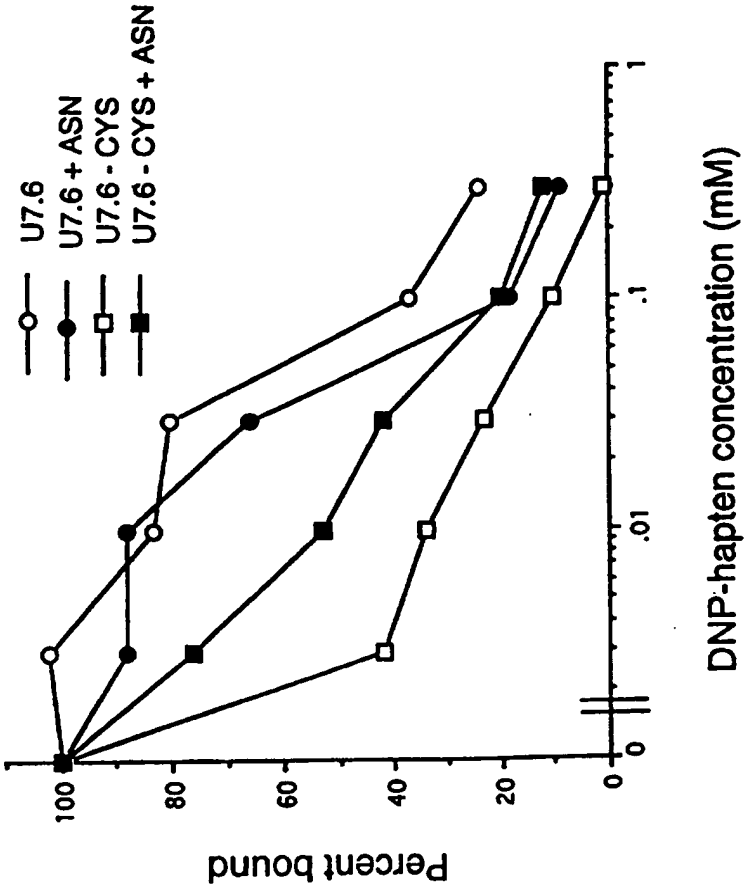

U7.6
U7.6 + ASN
U7.6 - CYS
U7.6 - CYS + ASN
Percent bound
100
80
60
40
20
0
0
.01
.1
1
DNP-hapten concentration (mM)

Figure 8

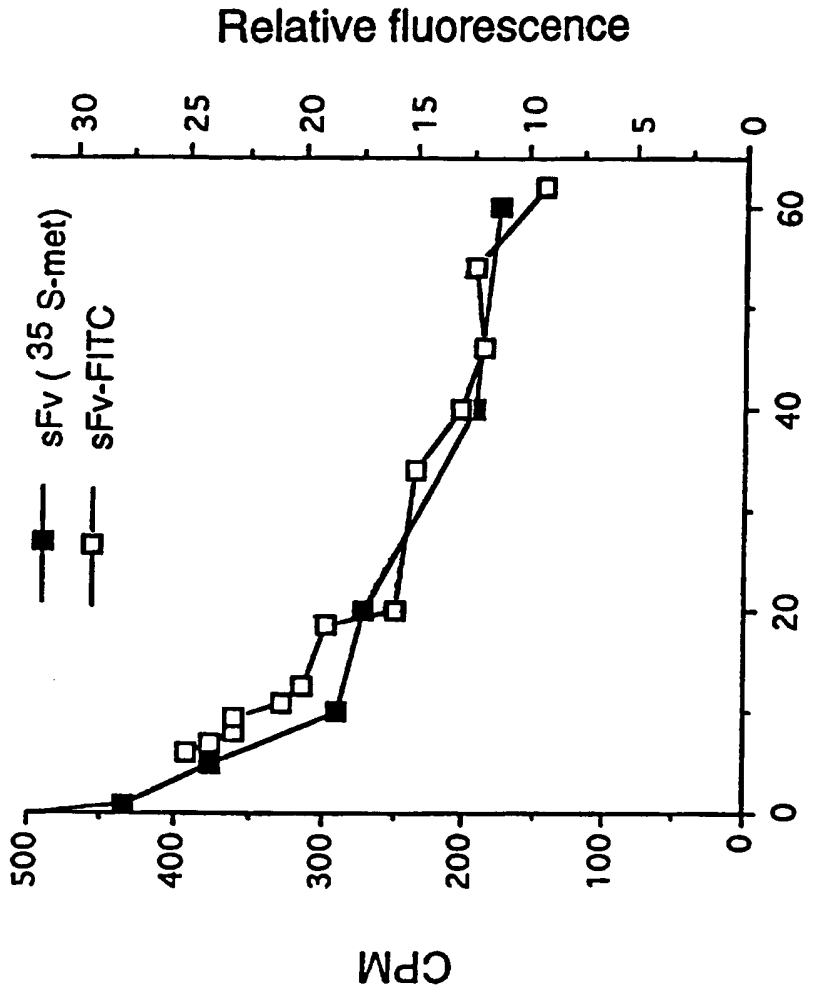

Relative fluorescence
30
25
20
15
10
5
0
60
40
20
0
Time (min)
sFv (35 S-met)
sFv-FITC
500
400
300
200
100
0
CPM

Figure 9

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 95/10348

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K16/00 G01N33/577

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF IMMUNOLOGICAL METHODS, vol. 165, no. 1, 27 September 1993 AMSTERDAM, NL, pages 81-91, P. NICHOLLS ET AL. 'An improved method for generating single-chain antibodies from hybridomas.' cited in the application see page 89, right column, line 18 - line 39 see abstract --- -/--	1-31

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

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Date of the actual completion of the international search	Date of mailing of the international search report
30 November 1995	29.12.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	Nooij, F

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INTERNATIONAL SEARCH REPORT

Int onal Application No
PCT/US 95/10348

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	THE JOURNAL OF IMMUNOLOGY, vol. 152, no. 4, 15 February 1994 BALTIMORE, MD, USA, pages 1802-1811, A. GEORGE ET AL. 'Redirection of T cell-mediated cytotoxicity by a recombinant single-chain Fv molecule.' see abstract see table 1 ---	1-31
A	CANCER, vol. 73, no. 3 suppl 1, 1 February 1994 PHILADELPHIA, PA, USA, pages 1105-1113, P. HAND ET AL. 'Potential for recombinant immunoglobulin constructs in the management of carcinoma.' see the whole document ---	1-31
A	WO,A,94 15642 (CREATIVE BIOMOLECULES INC. ET AL.) 21 July 1994 see figure 3 ---	6
P,X	THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 42, 21 October 1994 BALTIMORE, MD, USA, pages 26267-26273, C. JOST ET AL. 'Mammalian expression and secretion of functional single-chain Fv molecules.' see the whole document ---	1-18, 20-31
P,X	WO,A,95 15769 (IMMUNOMEDICS INC.) 15 June 1995 see examples see claims -----	1-5, 8-18, 20-30

1

INTERNATIONAL SEARCH REPORT

information on patent family members

Inte ɔnal Application No
PCT/US 95/10348

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9415642	21-07-94	AU-B-	6084694	15-08-94
		CA-A-	2153568	21-07-94
		EP-A-	0679093	02-11-95
WO-A-9515769	15-06-95	US-A-	5443953	22-08-95
		AU-B-	1331995	27-06-95